

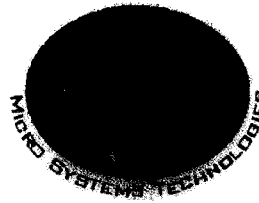
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February 21, 2007

OPPT Document Control Office (DCO)
EPA East Building (ICC)
1201 Constitution Avenue NW, Rm #6428
Washington DC 20004 (202) 564-8930
Attn: Audrey Binder and Mr. Alwood

Dear Ms. Binder and Mr. Alwood:

Please consider the enclosed as a premanufacturing notification of our intent to produce and demonstrate genetically modified microorganisms to the Department of Defense (DoD), Center for Disease Control (CDC), Environmental Protection Agency (EPA), Department of Energy (DOE), Department of Transportation (DOT), and different major contractors (Systems Research Development Corporation (SRDC), Northrup Grumman, Boeing, Cartwright, etc.) to qualify for specific projects. The research leading up to these demonstrations is being conducted in collaboration with scientists at Purdue University and Micro Systems Technologies. Micro Systems Technology, Purdue personnel will be conducting the demonstrations/tests. There is no direct government involvement in the demonstration, but different documents (i.e. SBIRs, solicitations) have been submitted to DOD and the Department of Energy on the usefulness of the technology.

We have put all the organism's development as well as the process application in the format of EPA's "points to consider" relative to TSCA field release applications. We hope you find this information satisfactory for your regulatory oversight. I will be the primary contact for questions concerning the demonstrations, application and demonstration unit, Dr. David Nivens (Purdue) (765) 494-0460 for the Bioreporter and demonstration unit. We look forward to a positive and productive review.

I thank you for allowing MST and Purdue University the opportunity to extend the demonstration/test period. We look forward to the approved TERA.

Sincerely,

Joseph F. Williams
President & CEO

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302914

**Toxic Substances Control Act (TSCA) Environmental Release Application (TERA)
for *Pseudomonas putida* (*P. Putida* TVA8).**

Micro Systems Technologies (MST), Dayton OH and the Purdue University, West Lafayette IN.

Reference: An initial TSCA Application was submitted on June 14, 1995 by the University of Tennessee and the US EPA Consent Order for Premanufacture Notice P95-1601 approved March 27, 1996. The initial TSCA was updated February 27, 2004 by Micro Systems Technologies and University of Tennessee, with the US EPA Consent Order for Premanufacture Notice TERA R-4-01 and 02, approved April 12, 2004. Other TERAs have been granted for use of other genetically modified *Pseudomonas putida* microorganisms. The US EPA has granted TERAs R-98-0004 and R-98-0005 to Oak Ridge National Labs for the use of genetically modified strains of *P. putida* (designations of R-01-0003 and R-01-0004) for use in the environment on a small site that allowed limited access.

Objective: The objective of this application is to (i) update approved TERA R-4-01 and, 02, (ii) update Premanufacture Notice P95-1601, as well as (iii) obtain an extension from the US EPA for the demonstration and continued testing of biosensor systems that use genetically-engineered, bioluminescent microorganisms. Specifically, the proposed testing will use *Pseudomonas putida* TVA8 to detect trichloroethylene in a series of environmental and off-site facility tests. *Pseudomonas putida* TVA8, has been genetically-engineered to producing light in the presence of TCE and some BTEX compounds. In our proposed tests, *P. putida* TVA8 will not be in direct contact with the environment, but instead enclosed within a sensor, with three levels of containment to dramatically minimize the possibility of any environmental release of the genetically-engineered organisms. The bioreporters are housed within a stainless steel bioreporter chamber, which in turn is sealed in a heating/cooling block. The entire system is contained within an enclosure, which houses the biomaterial, control and power circuits, airflow and photonic subassemblies. The sensor detects extreme low levels of TCE, producing bioluminescence, quantifies the amount of light produced by the strains, and sends a warning signal via a variety of communication technologies. For the environmental test tentatively scheduled for the United States Air Force, the sensor will be placed on the ground or suspended by a device, within a remote area of a military test site and exposed to TCE by a release system. For demonstration in buildings, a calibrated amount of TCE will be released into a sealed Heating Ventilation Air Condition (HVAC) enclosure housing the sensor unit.

Background: This proposed investigation seeks to continue testing the hypothesis that living bioluminescent signaling microorganisms (bioreporters) can be genetically engineered to sense and report the presence of chemical contaminants. Bioluminescent bioreporters typically contain a regulatory system that specifically interacts with a targeted analyte(s) and a promoter-*luxCDABE* genes fusion. The interactions between the analyte, regulatory protein, and promoter act as a switch that triggers the expression of the *luxCDABE* genes, which code for bioluminescence-producing proteins (see Appendix II, Nivens et al.,).

The original TSCA application allowed the direct release of a genetically modified microorganism (GEM) into a contained soil environment (lysimeter) to monitor bioremediation processes (see Appendix II, Ripp et al., Environ Sci. Technol. 34(5):846-853). In this study, the

GEM, *Pseudomonas fluorescens* HK44, was applied to the soil to monitor the degradation of naphthalene. The updated TSCA, approved on April 12, 2004 (TERA R-4-01 and 02), allowed the use of a *P. fluorescens* HK44 (list) to be sealed and used to monitor for naphthalene and/or methyl salicylate.

To further advance these bio-sensing applications, the current investigation seeks to seal a GEM within a new biosensor format and demonstrate the enhanced analytical performance of this biosensor when it is exposed to trichloroethylene. Applegate et al. has demonstrated that the *Pseudomonas putida* TVA8, containing a *todX::luxCDABE* fusion, produces bioluminescence in the presence of minute concentrations of solvents such as trichloroethylene, toluene, and benzene (see Appendix II, Applegate et al., Environ Sci. Technol. 34(5):846-853). This GEM, *P. putida* TVA8, was constructed from a benign parent strain, *P. putida* F1. Past approval for other *P. putida* strains has been granted to Oak Ridge National Labs (TERAs R-98-0004 and R-98-0005). In these studies, *P. putida* strains were directly released in the environment on a small site with limited access. The strains were given the tracking designations of R-01-0003 and R-01-0004.

In this study, the demonstration sensor will contain (i) a bioreporter sustain within a seal chamber, (ii) extremely sensitive photo-detector capable of detecting bioluminescence from the bioreporter organisms, (iii) unique air flow system, (iv) signal circuitry for quantifying the bioluminescent response, and (v) remotely communicating results to a dedicated receiver unit. To reduce risk of environmental contamination, the device has three levels of containment described in this document. Information on the strain, the device and tests are described in this document.

A. Description of recipient and donor microorganisms (information below updates the initial submitted and approved Premanufacture Notice TSCA document)

A.1. Taxonomy

A.1.a. The donor organism was *Escherichia coli* S17-1(λ pir) containing transposon mini-Tn5Kmtod-lux on plasmid pUTK214. The recipient organism was *Pseudomonas putida* F1, a natural isolate capable of degrading numerous toxic compounds, including toluene, benzene, ethylbenzene, and trichloroethylene. The recombinant strain is referred to as *Pseudomonas putida* TVA8 (Applegate et al. 1998. Appl. Environ. Microbiol. 64:2730-2735).

A.1.b. Identification of strain F1 as *Pseudomonas putida*, biotype B, was done by phenotypic testing (Gibson, et al. 1968. Biochemistry 7:2653-2662) coupled with 16S rRNA sequence analysis, which was carried out with the aid of the Ribosomal Database Project II web server, release 9.42. The 16S sequence analysis was able to adequately distinguish strain F1 from all bacterial species other than *P. putida* and its close relative *Pseudomonas plecoglossicida*. Strain F1 was identified as *P. putida* rather than *P. plecoglossicida* on the basis of the ability of strain F1 to grow on L-tryptophan and L-kynurenine (Gibson, et al. 1968. Biochemistry 7:2653-2662; Nishimori et al. 2000. Int J Syst Bacteriol 50:83-89). The 16S sequence of *P. putida* strain F1 is available from GenBank (accession number L37365).

A.2. Genotype

A.2.a. Natural transformation has not been described in *P. putida*. The transposable element described in this application (mini-Tn5Kmtod-lux), which is inserted into the genome of *P. putida* TVA8, lacks a transposase, preventing it from self-transposing to any transmissible plasmid that could potentially be acquired by *P. putida* TVA8 in the field. Mini-Tn5Kmtod-lux is stably maintained in the chromosome of *P. putida* TVA8 regardless of the presence of kanamycin selection. No plasmids, insertion elements, or transposons have been described as natural residents in *P. putida* TVA8 or *P. putida* F1.

E. coli S17-1(λ pir) carries two known transmissible genetic elements, the broad host range self-transmissible plasmid RP4-2-Tc::Mu-Km::Tn7 (Simon et al. 1983. Bio/Technology 1:784-791), which encodes resistance to trimethoprim and streptomycin, and λ pir (Kolter et al. 1978. Cell 15:1199-1208). Both elements are integrated into the chromosome of *E. coli* S17-1(λ pir). There was a potential for RP4-2-Tc::Mu-Km::Tn7 to transfer itself to *P. putida* F1 during conjugation and to be stably maintained in *P. putida*. However, no plasmid has been detected in *P. putida* TVA8. Bacteriophage λ is not known to be capable of injecting DNA into any *Pseudomonas* strain. Therefore, the probability of λ pir having been transferred itself or other *E. coli* genes to TVA8 is negligible.

A.2.b. Natural genetic exchange between *E. coli* and *Pseudomonas* is well-documented, particularly for clinical isolates. There are several incompatibility groups of plasmids that are maintained stably in both species.

A.3. Phenotype

A.3.a. Mini-Tn5Kmtod-lux contains a *tod-lux* reporter in which a DNA fragment containing the *tod* promoter of *P. putida* F1 is fused to a promoterless *luxCDABE* cassette derived from *Vibrio fischeri*. The reporter is preceded by a strong transcription terminator and followed by a kanamycin resistance gene. The above construct is flanked by O and I elements from Tn5. Mini-Tn5Kmtod-lux was cloned into the suicide vector pUT to generate pUTK214, which is carried by the donor strain, *Escherichia coli* S17-1(λ pir). *Escherichia coli* S17-1(λ pir) was used to transfer pUTK214 to *P. putida* F1 by means of conjugation, enabling mini-Tn5Kmtod-lux to transpose from pUTK214 into the chromosome of *P. putida* F1, thereafter referred to as *P. putida* TVA8. The pUTK214 plasmid is not able to replicate in *P. putida*, and is therefore not present in *P. putida* TVA8. The *tod-lux* reporter element of mini-Tn5Kmtod-lux allows *P. putida* TVA8 to produce light in response to any inducer of the *tod* promoter, including trichloroethylene. The transcription terminator upstream of *tod-lux* prevents *P. putida* promoters upstream of the mini-Tn5Kmtod-lux insertion site from regulating light production independently of *tod*. *P. putida* TVA8 retains the ability to degrade all substrates that are degraded through the *tod* gene cluster of *P. putida* F1, including toluene and trichloroethylene.

A.3.b. The *P. putida* F1 strain will grow on standard minimal salts media supplemented with simple carbon sources. They are obligate aerobes. They grow well between 20 - 30°C, and over a pH range of 5-8. Their optimum growth rate is at 25°C in rich, undefined media, with a doubling time of approximately 30 minutes.

A.3.c. *Pseudomonas putida* is not considered a pathogen, or to have any associated toxicity.

A.3.d. The lifecycle of TVA8 is typical for a strain of *Pseudomonas putida*.

A.3.e. *Pseudomonas putida* strain F1 was isolated from soil (Gibson et al, 1968. Biochemistry 7:2653-2662).

A.3.f. *P. putida* TVA8 has resistance for kanamycin. It produces no known antibiotics.

Also, see B.3.d. for information on plasmid maintenance in the engineered strains.

A.3.g. *P. putida* strain TVA8 is killed easily with standard disinfecting solutions.

A.3.h. As with other strains of *Pseudomonas putida*, TVA8 is expected to be prey for microeukaryotes in the soil. HK44 is also subject to predation by *Myxobacteria spp.* (B. Lampson, unpublished results).

B. Construction of the recombinant microorganism

B.1. Source and function of DNA used to modify recipient.

B.1.a. The DNA both plasmid and chromosomal was isolated according to the procedures described in Maniatis et al. (Molecular cloning: a laboratory manual, 1982). The DNA was identified after agarose gel electrophoresis and ethidium bromide staining.

B.1.b. *Pseudomonas putida* strain TVA8 is a genetically engineered microorganism that will be used in the proposed demonstrations/tests. This strain was developed as a bioluminescent reporter to measure *in situ* toluene /trichloroethylene catabolic gene expression. The basis for this trait is mini-transposon mini-Tn5Kmtod-lux, which contains the only genetically engineered DNA in the strain. This work was supported by the US Geological Survey and the Air Force Office of Scientific Research.

B.2. Vector construction

B.2.a. A complete description of the pUTK214 vector plasmid and the associated mini-Tn5Kmtod-lux transposon is given in the following paragraph. The genetic map of mini-Tn5Kmtod-lux, showing the origin of DNA fragments.

Ancestry of genetic elements in strains used for construction of the GEM. Mini-Tn5 transposons consist of any DNA sequence flanked by Tn5 O and I sequences. Mini-Tn5Kmtod-lux contains a transcription terminator, followed by a lux cassette under the control of the tod promoter, followed by a kanamycin resistance gene (Applegate et al, 1998. Appl. Environ. Microbiol. 64:2730-2735).

The lux genes were originally isolated from *Vibrio fischeri* strain MJ-1 (J. Engebrecht et al., Cell 32:773-781, 1983) and cloned into pUCD5B to create pUCD615 (Rogowsky, et al, 1987. J. Bacteriol. 169:5101-5112). The luxCDABE gene cassette, carried on a 8.35 kb KpnI-PstI fragment, was cloned from pUCD615 into pLJS (Applegate et al, 1998. Appl. Environ.

Microbiol. 64:2730-2735), allowing it to be cloned as an *Xba*I-*Pst*I fragment into pUC18-Not. The resulting plasmid was designated pUC18-Not-*lux*.

A 1.8 kb *Sma*I-*Xho*I DNA fragment containing the *todR* pseudogene, *tod* promoter, and the 5' end of *todX*, originally isolated from *Pseudomonas putida* F1 (Menn et al, 1991. Gene 104:91-94), was subcloned into pLJS, allowing it to be cloned as a *Spe*I-*Xho*I fragment upstream of *luxCDABE* in pUC18Not-*lux*, so that the *lux* operon is transcribed from the *tod* promoter. The resulting plasmid was designated pUC18Not-*todlux*.

The commercial expression vector pKK223-3 (GenBank accession number M77749) includes a 0.4 kb DNA fragment encoding a *rrnB* 5S rRNA gene and its two tandem transcription terminators, originally isolated from *Escherichia coli*. A 0.8 kb *Hind*III-*Hinc*II fragment encompassing the 0.4 kb fragment was cloned from pKK223-3 into pLJS, yielding plasmid pLJST2.

The transposon mini-Tn5Km*NX* was constructed by PCR amplification of a kanamycin/neomycin resistance gene and its promoter (Beck et al, 1982. Gene 19:327-336, original source: transposon Tn5, first isolated from *Klebsiella pneumoniae*) from the cloning vector pCRII (Invitrogen Corp, Carlsbad, CA). The PCR primers were used to create *Xba*I and *Not*I restriction sites upstream of the promoter, as well as to create the flanking Tn5 O and I sequences that define the termini of the mini-transposon (Applegate et al, 1998. Appl. Environ. Microbiol. 64:2730-2735).

The suicide transposon delivery vector pUT (Herrero et al. 1990. J. Bacteriol. 172:6557-6567) is a plasmid which carries an ampicillin resistance gene, a transposase gene derived from transposon Tn5, the origin of replication of plasmid R6K, and the origin of transfer of plasmid RP4. pUT has a restricted host range and is unable to replicate in *Pseudomonas*.

Plasmid pUTK214 was assembled as follows: mini-Tn5Km*NX* was cloned into pUT to create plasmid pUTK210. The 0.8 kb transcription terminator DNA fragment was cut out of pLJST2 with *Not*I and *Avr*II and cloned into the *Not*I-*Xba*I site upstream from the kanamycin resistance gene promoter in pUTK210, creating pUTK211. This cloning step left the *Not*I site intact and introduced a new unique *Xba*I site that originated in the multicloning site of pLJS. Both sites were located between the transcription terminator fragment and the kanamycin resistance gene promoter. The *tod* promoter-*lux* cassette fusion was cut out of pUC18Not-*todlux* as a *Not*I-*Xba*I fragment and cloned across the *Not*I-*Xba*I sites of pUTK211, creating pUTK214.

The complete nucleotide sequence of the *lux* gene cassette (T.O. Baldwin *et al.*, J. Biol. Chem. 264:326-341, 1989) has been determined and published.

B.3.a. Introduction of the vector to the recipient was done by bi-parental mating followed by selection on *Pseudomonas* isolation agar containing kanamycin, with confirmation toluene-inducible light inducible response and retention of ability to grow on toluene as a sole carbon and energy source. Ampicillin sensitivity was ascertained in order to confirm that the pUT-derived portion of pUTK214 was not retained by *P. putida* TVA8.

B.3.b. Mini-Tn5Km*tod-lux* is stable in *P. putida* TVA8. Batch assays were performed by growing TVA8 on toluene as the sole carbon and energy source and transferring a 1% inoculum

to fresh medium every day for five days. Plate counts were performed on media with and without kanamycin. The ratio of CFUs on selective medium compared to non-selective medium was 1.12 ± 0.13 , indicating that loss of mini-Tn5Kmtod-lux from the TVA8 population during the experimental time frame could not be detected. Colony hybridizations with a *luxA* DNA probe supported this conclusion. A continuous culture stability study run for 100 generations also failed to detect loss of mini-Tn5Kmtod-lux from the TVA8 population, yielding a selective /non-selective plate count ration of $1.05 \pm .13$ (Applegate et al, 1998. Appl. Environ. Microbiol. 64:2730-2735).

C. Characteristics of the recombinant microorganisms

C.1. Phenotype

C.1.a. There are two principal means of identifying strain TVA8 in comparison to its parent strain F1. TVA8 is kanamycin resistant, while the parent is not. TVA8 produces visible light when in the presence of toluene, trichloroethylene, and certain other organic solvents, while the parent does not. Molecular techniques (see below) can also be utilized.

C.1.b. TVA8 will grow on standard minimal salts media supplemented with simple carbon sources or with toluene. It is an obligate aerobe. They will grow well between 20 - 30°C and over a pH range of 5-8. Their optimum growth rate is at 25°C in rich, undefined media with a doubling time of approximately 30 minutes. No other factors distinguish these strains from responses seen in wild-type bacteria.

C.1.c. Background research has been conducted on the bioluminescent trichloroethylene sensing characteristics of reporter strain TVA8. Log phase cells incubated for 3 hours in minimal salts medium with trichloroethylene gave a bioluminescent response to trichloroethylene with a detection limit of 5 µM (Shingleton et al, 1998. Appl. Environ. Microbiol. 64:5049-5052). The bioluminescent response was accompanied by an increase in mRNA transcript levels of both *luxA* and *todC1*. The bioluminescent response to trichloroethylene concentration was linear up to a trichloroethylene concentration of 80 µM.

C.1.d. TVA8 will grow on standard minimal salts media supplemented with simple carbon sources or with toluene. No other cofactors are needed. TVA8 does not form spores. It can be easily killed using autoclaving or common chemical agents (e.g. bleach).

C.1.e. The antibiotic resistance for TVA8 includes kanamycin, neomycin, and related aminoglycosides.

C.1.f. Strain TVA8 can be detected using conventional agar isolation plates supplemented with kanamycin (50 mg/l). Limits of detection are at least 1 cfu/gram soil or /liter water. Molecular techniques can also be used for the identification of this strain. DNA probe hybridization can detect as few as 10^5 bacteria; PCR amplification can detect as few as 1 bacterium. The detection limits are independent of sample size, although 100 g soil or 1 liter water samples are common. Molecular techniques utilize the unique *lux* sequence for positive identification. In addition, bioluminescence based most probable number (MPN) techniques can be used to detect

bioluminescent bacteria like TVA8 (see Appendix II, Ripp et al., 2000, Appl. Microbiol. Biotech. 53:736-741).

C.3. Toxicity Testing

C.3.a. No toxicity or pathogenicity (apart from opportunistic infection) is known to be associated with these microorganisms or their parent microorganisms.

C.4. Possible Environmental Effects

C.4.a. No known effects on biogeochemical processes are associated with these microorganisms.

C.4.b. The frequency of genetic exchange between these microorganisms and other microorganisms under in situ conditions is unknown. Data from long-term lysimeter, lab microcosm, and chemostat studies suggests, however, that gene transfer frequencies are extremely low and below current limits of detection.

C.4.c. *Pseudomonas* species are environmentally ubiquitous. The addition of these recombinant microorganisms is not predicted to deleteriously affect indigenous organisms.

C.4.d. No other organism is a target for effect by HK44 or 5RL.

D. Production process and worker exposure

D.1. Production Process.

Frozen stocks of cells will be maintained at Purdue University, West Lafayette IN. At Purdue, cells will be grown in minimal salts medium and aliquots of the culture containing 10^8 cells will be transfers to the biomaterial chambers (316 stainless steel) using sterile techniques. The biomaterial chambers are then tested and secured in sealed packages and shipped overnight to their final destinations for testing.

D.2. Containment

D.2.a. The facility has been designed for maximum physical containment with biosafety level 1 and 2 perimeters. Purdue University follows the NIH guidelines for recombinant DNA use and safety protocols are in place (IBC #05-007).

D.2.b. In case of accidental release of the culture, the spill area will be disinfected with chemical agents such as sodium hypochlorite (Bleach). Complete destruction of will be determined by standard plating techniques.

D.3. Production volume

D.3.a., b. A 10 to 50 ml culture will contain approximately 1×10^8 viable *P. putida TVA8* cells per ml will be prepared and 1 to 10 ml volumes will be filtered onto a porous matrix and the matrix will be transferred to a sterile biomaterial chamber.

D.4. Worker exposure during production

D.4.a. Only workers skilled in the handling of cell cultures will participate in this phase of the project. Standard laboratory safety practices will be followed. After the GEMs are sealed in the device, the risk of exposure is extremely minimal, because the GEMs are contained within a device and do not directly contact the environment.

As per our safety protocols, gloves and other safety equipment will be use by the skilled workers during all interactions with the cells and standard sterile techniques will be utilized.

E. Environmental release protocols

E.1. Objectives

E.1.a. The objective of this advanced research and demonstrations project is to generate data to aid in the development of an inexpensive sensor device with the capability to provide early detection of harmful chemical agents. This autonomous sensor technology has the potential to support the missions of many different Federal, State, and Local government agencies.

The specific intent of the investigation is to demonstrate that bioluminescent reporter bacteria (*P. putida* TVA8) sealed within a device can be sued to sense TCE. These demonstrations are to determine if this bio-reporting chemical sensor can be used in application outside of the laboratory. Field demonstrations will employ the use of automated building monitoring systems, stand-alone sensor systems, unmanned aerial/ground vehicle technologies (on DoD approved test ranges), non-research facilities (application development facilities; i.e. Lockheed Martin, Boeing, SRDC, etc.) and ground remote device technologies (on DoD approved test ranges and contractor facilities). All demonstrations provide microorganism health monitoring and near real-time reporting of agent detection, identification and quantification information.

The broad goal of these demonstrations/advanced research tests is to develop multi-user specific demonstrations/tests for monitoring target agents (current chemical analytes - TCE and methyl salicylate) in the environment and facilities that have and have not undergone decontamination.

The proposed demonstrations/tests are designed specifically to examine the ability of GEMs in detecting and monitoring TCE via bioluminescent signaling in air and potentially water. The inclusion of the recombinant strain is obviously vital to the success of the project.

E.1.b. We have identified few risks associated with this project. There are no pathogens or pathogenic genes involved in this work. The *P. putida* TVA8 cells are sealed in a device with three stages of containment. First, the GEM is contained in a stainless steel chamber equipped with 0.2 μm pore size matrices with minimal growth medium. Second, the stainless steel chamber is contained within an aluminum heating/cooling block and interfaced to an air flow subassembly also equipped with a 0.2 μm pore size filter. Third, the entire subassembly is then house in an enclosure, which also contains the electronics. Since the GEMs are housed within the sensor, actual direct contact with the outside environment is eliminated, risk associated with this testing will be minimal to nonexistent.

In the event of release after a catastrophic event the bacteria are generally regarded as harmless. There is no special growth advantage of this strain outside of the analyte contaminated area. In a previous field release study, strain HK44 was found to exhibit no detrimental environmental effects. In laboratory soil microcosm and chemostat studies, strain 5RL was shown to exhibit no detrimental effects on competing populations.

Prior to field demonstrations, the sensor technology, coordinated through the United States Air Force, will go through validation testing by the National Center for Toxicology Research (NCTR – FDA's facility in Jefferson Arkansas). This validation testing is currently scheduled for late February 2007.

E.1.c. Demonstrations (Field Test, Ft Carson Air Burst Range and at Systems Research and Development Corporation (SRDC), Palm Beach Gardens FL) are tentatively scheduled during the March 2007, with data collection/analysis occurring with every test. The duration of the requested 24 demonstrations / 60 tests are expected to take three years from TSCA approval.

E.2. Nature of the site

E.2.a. Several scheduled demonstrations with current prototype (design #4) will be conducted at SRDC's facility and in remote operated areas of military test ranges and in a military special water testing tank. Other scheduled demonstrations will be in government facilities by the use of test chambers and automated building systems. Local city governments are also interested in a demonstration to better understand real-time autonomous hazardous monitoring. In addition, the sensor device will be mounted onboard UAVs and tested on a military test range. With TSCA approval, air, water and ground testing will occur with a microorganism installed in current demonstration device, prototype#4, configuration to ensure the established risk process validates the safety design precautions. Risk mitigation is based on the principles that risk management must be forward looking, structured, informative, and continuous. After these successful validation and field tests an aerial demonstration will be scheduled.

The United States Air Force Reserve utilizes a specific area of Fort Carson for pilot qualification training. This area is approximately 50+ acres, and contains a range control tower, range personnel facility and specific designated impact areas for aircraft weapons. This site was chosen because it is uncontaminated for the specific agents, has controlled access from the rest of Fort Carson test areas and the civilian population, accessible to the range personnel only, and reasonably secure.

Only MST or Purdue personnel will handle any microorganisms outside of the laboratory. The UAV carried tests parameters have been coordinated with Systems Research and Development Corporation (SRDC), Palm Beach Gardens FL and the Fort Carson Range Commander/Test Range Director. Any flight test with or without the microorganism will be for a maximum flight duration of 3 hours.

The current version of the Bioluminescent Bioreporter Integrated Circuit (BBIC) sensor demonstration device will be used for validation and field tests. The sensor system will transmit real-time data from the sensor unit to a PC in the range tower and to Wright-Patterson Air Force Base. The sensor system will be placed on two specific areas on Ft Carson (along side a

dirt/sandy road and hidden with trees). There will be four sensors will be placed on the test range within five meters of each other for each test. Metrological data will be used each day to better understand detection anomalies. This release system will manage the amount of dispensed TCE, timing of release and safety of containment.

After the Premanufacture Notice TSCA approval, ground testing will occur with a microorganism installed in the demonstration device (prototype #4) to validate the safety design established by the risk mitigation process. Risk mitigation is based on the principles that risk management must be forward looking, structured, informative, and continuous.

After successful ground tests at Ft Carson, SRDC, government facilities, local City test areas, or any designated site by the customer, MST will submit a copy of the test report submitted to the Customer. This report will cover as a minimum the EPA Hqs TERA test information.

Test parameters for demonstrating the usefulness of the BBIC technology for water and soil contamination tests have not been developed for this Premanufacture Notice TSCA request. Several government agencies are looking at the BBIC technology and its efficacy for a portable leave behind device or a Hazard Monitoring Real-Time System. Tests using SRDC's UAV onboard equipment configured for a portable device with BBIC technology is complete. The following being developed for a demonstration for the following agencies:

- (1) Special Forces – detection / defeat (Tri-Service)
- (2) Real Time Hazard Identification Warning System for specific cleanup sites, FERNALD (DOE)
- (3) STRICOM for Dugway Proving Grounds (Army)
- (4) Chem/Bio Training (Air Force Research Laboratory)
- (5) Force Protection Requirements (Air Force Protection Battlelab)
- (6) Submarine/ship air quality & base water/stream/river contamination monitoring (Navy)

Only individuals skilled in the handling of bioreporters will participate in the installation and removal of the biomaterial in the demonstration sensor device for this phase of the project. Standard approved practices will be followed during bioreporter monitoring system use.

E.2.b. Another demonstration of the biosensor device will be inside a government facility, DOE FERNALD project. With TSCA approval, a test will occur to detect TCE inside a facility selected by DOE. The test will demonstrate a Real-Time Hazard Monitoring System will detect extreme low levels (ppt) of TCE and provide an alarm/concentration to an automated building management system, contact First Responders, Fire Department, Security (including police), and control HVAC, security locks, lighting and security cameras, as required.

After validation and field testing MST will contact Homeland Security to demonstrate the sensors ability within a cargo shipping container. After successful ground tests the demonstration prototypes will be mounted internally, near the container door opening, and be connected to a data recorder. The data recorder is expected to provide power to the sensor, data recorder and a tiny light on the outside of the container. Detection and quantification data will be recorded

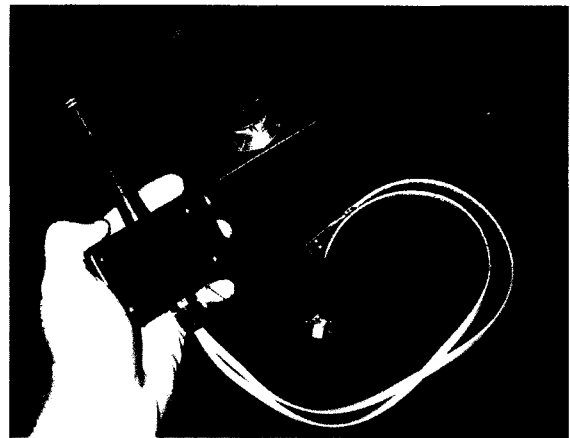
during the demonstration. The demonstration is to detect trace amounts of analyte (i.e. selected to not harm container cargo) in a container and produce a tiny light protruding through the side of the container to indicate the sensor detect analyte in the container. The detection sensor units will be removed and shipped to Purdue University for further analysis.

Test parameters for other air, water and soil demonstrations have not been developed at the time this TSCA premanufactured notice request was developed.

E.2.c. At the immediate conclusion of each demonstration, the encapsulated bioreporters will be disposed of directly at the field site by immersion in a 10% bleach solution contained within a leak-proof container or returned to Purdue University for analysis and then destruction by proper approved methods.

E.3. Demonstration/test device design

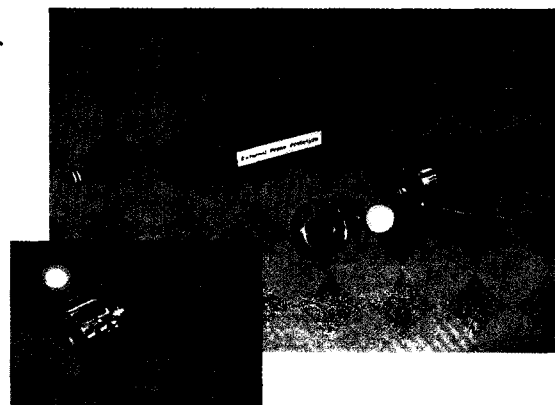
E.3.a. The following information should provide a "General Description" of the Sensor Technology unit. There are four versions of the sensor device. Initial version (right) was designed to demonstrate the technology outside the laboratory. It has been tested in an unmanned air vehicle configuration, laboratory, and concealed areas (outside of UT laboratory). This design allowed the sensor device to be placed anywhere within a specific distance from the RF PC unit.



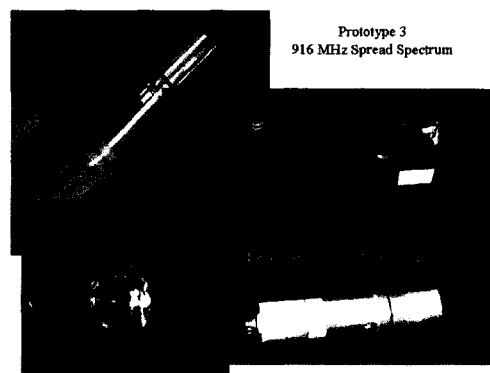
The second version (below) is designed for water and areas requiring a wand (to reach different depths, deep cavities [ground/air vehicle], HVAC, etc.).

Each "Sensor Detection Unit" (prototype #2, 3 and 4) power source is either battery or tied directly into the system power generation system. The communication system is not limited to a single type. The Sensor Detection Unit has transmitted data by SATCOM interface, RF (IR, 918 MHz, Spread Spectrum), fiber optic, and wire technologies. The Bioreporter Chamber, Airflow Chamber and Photosensor Subassemblies are the heart of prototype #4. The microorganism sensitivity levels for each specific agent have been engineered to detect in the part per trillion levels by a unique interface. A power supply/controller Subassembly regulate power, monitor Subassemblies and provide management for the sensor data. All these parts are housed in a hardware box. The software used to illustrate the data from the sensor detection device is LabView (COTS).

The second version device will have the basic design of version one except the stainless steel cylindrical w/welded cap and threaded mount is not part of the design. A wand is provided that will house the bioreporter, OASIC, connector and wire assembly. All other parts listed above will be part of this device. The length of the wire (fiber optic or wire) is determined by the user needs.

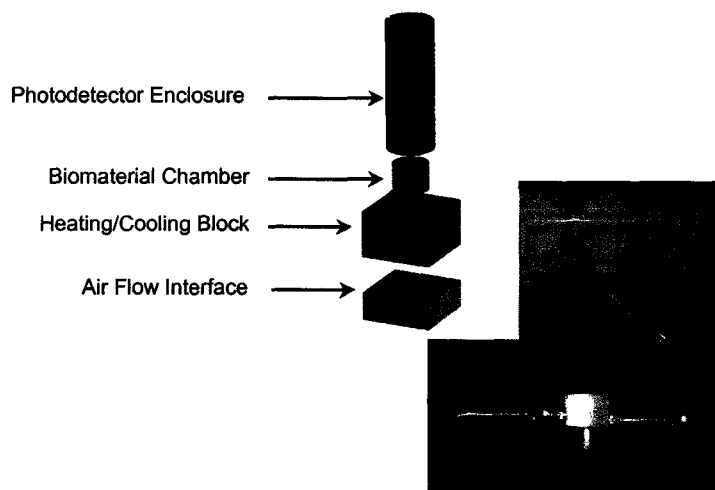


The picture to the right is the third version of a sensor demonstration device. The one major difference from the second demonstration version is the use of Spread Spectrum communication technology.



The current version of the sensor technology has five subassemblies (i.e. Airflow, Biomaterial, Photosensor, Power and Enclosure). The communication technology is an external part of the sensor device since the sensors output is able to be transmitted by many types of communication technology.

E.3.b. Pictures of early prototype demonstration devices and UAV mounting are included. Prototype #4, seen here, is the current design that will go through validation and field testing with the Air Force and the Department of Energy. In addition, the enclosure, power supply and monitoring electronics, and a portion of the Airflow Chamber subassemblies are not shown here.



E.3.c.

Sample Type	Sample Location
air	The sensor will be either placed on the ground, suspended in the air, placed inside a facility, on a building/tower/table, mounted inside Unmanned Air Vehicle (UAV) or outside the Unmanned Ground Vehicle (UGV). The demonstration device is design to be left unattended. Sensor detection data is transmitted by commercial or military communication technologies.
soil	The sensor can measure analyte either by placement near/around a contaminated soil area or a sample of the soil will be collected and placed into a container and connected to the sensor to allow the vapor to be drawn into the Biomaterial Chamber Subassembly.
water	A new sensor enclosure would be required for water testing.

E.4. On-site Containment Practices

E.4.a. After production of the 1 ml bacterial culture, the cells will be washed in a minimal medium to starve the cells and then resuspended at a concentration of approximately 10^8 /ml. A 0.1 ml aliquot of this culture will then be combined with an appropriate encapsulation matrix (alginate, PDMS, latex). The encapsulated cells will then be sealed in a 0.45 micron filter unit and transported to the test site in double sealed containers held at 4°C in labeled coolers.

This work will be performed by one-two persons skilled in handling bacterial cultures with appropriate safety training.

E.4.b. Instruments and tools used for manipulating the GEM will be considered contaminated. After use they will be stored inside closed plastic bags or in closed containers for transport to the laboratory where they will be sterilized either by autoclaving or by chemical treatment.

E.4.c. In accordance with standard operating procedures at Purdue University for the disposal of genetically engineered organisms, all contaminated sample material will be autoclaved or chemically treated until the contents are sterile.

E.4.d. Biological containment procedures will not be used for these experiments.

E.4.e. The Fort Carson site is located on property owned by the U. S. Department of Defense. As such, access to the site is limited to authorize personnel. USAFR range personnel and Army security personnel maintain this limited access.

E.4.f. The military ranges will undergo an inspection prior to any demonstration/testing. These inspections will be performed by USAFR range, Department of Environmental Compliance and Management managers and personnel performing the demonstration/test from MST and Purdue. Details of this testing procedure are available upon request. Only specific personnel will be allowed to participate in this phase of the demonstration.

E.4.g. Although the measurements will be made with the sensor device and Gas chromatography equipment, the site will also be examined daily while the demonstration/tests are ongoing. The site and equipment will be visually inspected for integrity and proper equipment function, as well as for any obvious abnormalities e.g. presence of wild animals. All demonstration parameters used for any type of test will be registered and carried to each test site. (Sample Observation Form, Appendix I).

E.5. Application Methods

Cell growth and encapsulation will be performed entirely within laboratory confines. Filter units containing the encapsulated cells will be transported to the test site in double sealed, leak-proof containers.

E.5.a. Gloves will be worn during all preparation and application procedures with the GEMs.

TCE is an environmental contaminant and must be disposed of properly.

A number of visitors are expected to witness the demonstrations/tests. These visitors will be kept at a safe distance from the test operations (minimum distance determined by Range Director and MST is 1000ft).

E.6. Termination and mitigation procedures

E.6.a. At the conclusion of the demonstrations/tests, the demonstration devices will be cleaned and packaged to be shipped back to Purdue for analysis. It is anticipated that the demonstration devices will be used in the future for other demonstration/tests.

E.6.b. In the event of a catastrophic failure, the demonstration/test will be terminated immediately. Evaluation of the sensor device biomaterial subassembly condition is the first priority after approval to enter the area is provided or by the test director and range director (military range authority) has ensured the test area is safe. Given the strength of the sensor device, biomaterial subassembly, damage to the biomaterial subassembly is very unlikely to happen. However, the porous material may be damaged by unforeseen events. In any event the biomaterial subassembly will be removed and replaced. The Environmental Management personnel for the test area will provide direction if an incident occurs. Increased area monitoring will be performed after any catastrophic event.

E.6.c. The test range environment will be protected during all tests by covering the ground with chemical absorbent pads. If adverse environmental effects are observed the area will be cleaned IAW with test range guidelines for TCE cleanup.

E.6.d. A monitoring plan will be developed to detect contamination in the unlikely event of a leak or spill. The sensor devices will be leak tested before shipment and before any test begins,

making subsequent leaks highly unlikely. Any damage to a sensor will require immediate delay or termination of the test. Demonstration personnel will be advised of the risks and the protocols involved in the job.

E.7. Sampling Procedure

E.7.a. Air sampling will be accomplished autonomously by the sensor device. The airflow subassembly is designed specifically for air monitoring (i.e. on the ground, carried in the air, placed in a room or cargo container, etc.). The measurement apparatus is permanently mounted in the demonstration device.

E.8. Record keeping and reporting test results

E.8.a. Test reports on the experiment will be produced during the demonstration/tests of the project. The reports will include, but are not limited to, the following: test parameters, operation evaluation, analysis of the data from sampling and monitoring, sampling schedule, and environment safety and health evaluation (including all accidents and injuries).

E.8.b. Progress report format will include, but is not limited to, the following: summary statement of progress to date, structured analysis of raw data (including statistical analysis and comparison to standard values), sampling schedule (including volumes), waste disposal schedule, and environmental safety and health report.

E.8.c. Reports will include test parameters for known scheduled demonstrations, locations and expected personnel to attend.

E.8.d. Information on bioluminescence sensing will be stored on computer files. Back-up files will periodically be produced and stored at a location separate from the main storage area. Daily procedures followed, as well as information gathered using molecular techniques or standard laboratory techniques will be stored at either the Food Science Department at Purdue University, Center for Environmental Biotechnology or Micro Systems Technologies.

E.8.e. All personnel involved in this project will follow standard laboratory and Test & Evaluation procedures. The details of these procedures will be kept in a written format that is available to all personnel. A copy of all training documents, laboratory practices, and necessary permits and certificates will be kept in the Food Science Department at Purdue University.

APPENDIX I

OBSERVATION FORM

Date:

Time:

Samples taken (if required):

Soil

Air

QC monitoring

Bioluminescence

Temperature

Other

Waste generation and disposal:

Health and Safety Concerns:

Risk Mitigation/Management

Other observations:

Signed: _____

Appendix II

The following documents will make up appendix II

Applegate TAV8 paper.pdf

Nivens et al, JAM 2004.pdf

Shingleton et al. 2001.pdf

Ripp et al. 2000.pdf

Quantification of Toluene Dioxygenase Induction and Kinetic Modeling of TCE Cometabolism by *Pseudomonas putida* TVA8

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Abstract: As measured by the toluene-induced bioluminescent response of *Pseudomonas putida* TVA8 in batch experiments, toluene dioxygenase (Tod) enzyme activities are dependent on toluene concentration between 0 and 30 mg/L. To provide a measure of the Tod activity for use in Michaelis-Menten competitive-inhibition kinetics, a correlation between toluene concentration and induced Tod activity as measured by an induced bioluminescent response of *P. putida* TVA8 is presented as a nondimensional Tod activity parameter. A packed-bed, radial-flow bioreactor (RFB) using the bioreporter *P. putida* TVA8A serves as the model system for studying the effect of the enzyme activity parameter on model predictions of vapor-phase toluene oxidation and trichloroethylene (TCE) cometabolism. Mass balances were performed on a differential section of the RFB to describe the radial transport of vapor-phase toluene and TCE through a bulk gas phase and the concomitant biological reaction in a stationary biofilm phase. The finite-element Galerkin weak-statement formulation with first-order basis functions was used to find the optimum solution to the highly nonlinear, coupled equations. For this RFB system with toluene concentrations less than 1 mg/L in the bulk gas phase, the Tod activity parameter enables accurate predictions of steady-state TCE degradation rate (0.27 $\mu\text{g TCE/min}$). © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* 76: 341–350, 2001.

Keywords: biodegradation bioluminescence; *Pseudomonas putida*; *tod-lux* bioreporter; toluene; toluene dioxygenase activity; trichloroethylene (TCE)

INTRODUCTION

Toluene dioxygenase (Tod) is a multicomponent enzyme system that is responsible for the metabolism of toluene

in *Pseudomonas putida* strains. Bacteria containing the Tod enzymes metabolize aromatic hydrocarbons such as toluene, benzene, *p*-xylene, and benzene derivatives with some, such as toluene, as sources of carbon and energy for cell function. By incorporation of two atoms of molecular oxygen, the same enzymes involved in oxidizing toluene to form *cis*-dihydrodiols are also responsible for oxidizing trichloroethylene (TCE) to form formic acid and glyoxylic acid (Lau et al., 1994; Li and Wackett, 1992). While TCE can nonspecifically induce the Tod system in some bacterial strains (Head and Jenkins, 1994; McClay et al., 1995; Shingleton et al., 1998); the oxidation of TCE does not support growth or energy production by the organism. Aerobic TCE degradation is thus considered cometabolic in that primary enzyme induction and physiological activity are at the expense of the natural substrate for the dioxygenase-initiated pathway for toluene catabolism. Thus, TCE cometabolism is a nonproductive competing process.

Problems with aerobic cometabolism of TCE include substrate competition, enzyme deactivation, and toxicity to the organism (Chang and Alvarez-Cohen, 1995; Kelly et al., 2000; Wackett, 1997; Yu, 1998). In order to overcome TCE-mediated enzyme deactivation and cellular toxicity, a primary carbon source such as toluene must be appropriately added to both induce the enzyme system and to balance substrate-competition effects with the necessary Tod activity and cell viability. That is, at toluene concentrations above 1 mg/L, the enzyme activity and cell viability are desirably high, but the concomitant, high competitive inhibition of TCE degradation is undesirable. This warrants the use of toluene feed cycles to eliminate the competitive inhibition between toluene and TCE for the Tod active site (Kelly et al., 2000). However, relatively low toluene concentrations in the feed stream could balance low competitive inhibition of TCE cometabolism with adequate Tod enzyme activity and biomass

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Contract grant sponsor: Waste Management Research and Education Institute, The University of Tennessee

concentrations. No known data exist on steady-state, vapor-phase TCE cometabolism using toluene concentrations less than 1 mg/L.

In conventional axial-flow, packed-bed bioreactors, the bed length is much longer than the bed diameter (height). Hence, the feed stream must contain a higher concentration of toluene to maintain Tod activity throughout the bed. In axial-flow beds, a relatively small region typically exists where competitive inhibition of TCE is small enough to allow substantial TCE degradation. In other words, most TCE degradation in steady-state, axial-flow, packed-bed bioreactors occurs only in a narrow cross section, which leads to wasted bed volume. However, a novel packed-bed, radial-flow bioreactor (RFB) first used by Rouhana (1997) for hazardous waste gas degradation using *P. putida* ATCC 23973 to degrade *p*-xylene was designed to have short bed length relative to the bed height (Fig. 1). TCE degradation can occur throughout the entire bed length, thereby eliminating both wasted bed volume and feed cycles by feeding low concentrations of toluene to sustain metabolism and enzyme production.

Bioluminescent bioreporters are used to indicate the state of organisms or to signal environmental changes in the surroundings. Examples include toluene concentration (Applegate et al., 1998; Kelly et al., 2000), TCE

concentration (Shingleton et al., 1998), specific growth rate (Huang et al., 1993), and Tod enzyme activity (Shingleton, 1998). With the advent of bioluminescent bioreporter organisms, the effect of environmental changes on a specific organism can be determined at the genetic level by measuring light responses.

Changes in genetic expression of the Tod degradative genes results in varying enzyme concentrations that ultimately determine the degradation rate of the toluene and TCE (Lau et al., 1994; Li and Wackett, 1992). However, current kinetic models used in bioreactor design do not account for variable enzyme concentrations within an organism. Furthermore, the analytical tools necessary to correlate such changes in cellular activities to changes in environmental conditions are either time consuming or nonexistent.

The objective of this study is to validate a new tool for measuring in vivo enzyme activities by first determining the mathematical correlation between toluene concentration and Tod activity as measured by a bioluminescent response of *P. putida* TVA8. Then, the correlation is shown to provide a kinetic model with the proper functionality to predict the performance of TCE cometabolism using steady-state feed concentrations of less than 1 mg toluene/L to a RFB.

MATERIALS AND METHODS

The strain used in this study is the bioluminescent bioreporter *P. putida* TVA8, a derivative of *P. putida* FI containing a *tod-lux* fusion on the chromosome (transposon mini-Tn5Kmtod-lux). The strain also contains the wild-type *tod* operon (Applegate et al., 1998).

Bioluminescent Response of *P. Putida* TVA8

The bioluminescent response of *P. putida* TVA8 over time to various concentrations of toluene was measured using a growing cell assay adapted from Heitzer et al. (1998, 1992). Cultures were prepared from a frozen stock of TVA8 by inoculating 1.0 mL of the stock solution to 100-mL yeast extract-peptone-glucose medium (YEPG) (Sayler et al., 1979) amended with 10 mL of a 50 mM phosphate buffer. The YEPG medium prevents energy limitations in the bioluminescent reactions. From a fresh overnight culture, a subculture was grown at 30°C to an optical density of 0.35 at 546 nm. Then, 2.0-mL aliquots of the culture were added to 20-mL scintillation vials containing 2.0 mL of a mineral salts medium (MSM) (Stanier et al., 1966).

Toluene was added by supplementing the 4-mL culture with known volumes of toluene saturated MSM. The volume of toluene-saturated MSM required for a given liquid phase concentration was calculated based on a mass balance. Gas-liquid equilibrium was predicted using Henry's law coefficient for toluene at 20°C (Chang and Alvarez-Cohen, 1995). The vials were placed in a

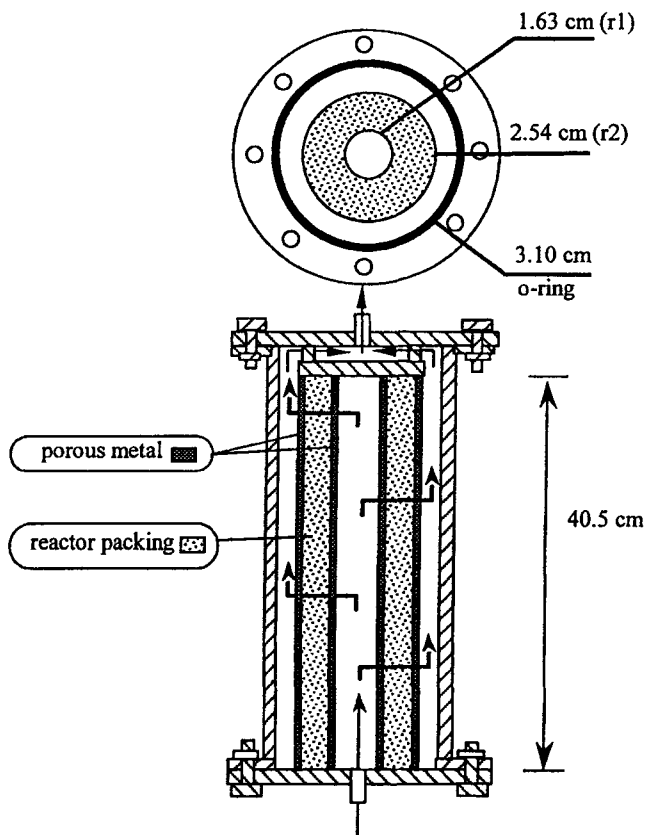


Figure 1. Cross section of the radial-flow bioreactor (RFB). $V_B = 483$ mL, $V_S = 332$ mL, $\epsilon = 0.0015$, $d_S = 0.025$ cm, $\delta = 0.0017$ cm, and $X_0 = 0.5$ mg cells/mL.

constant-temperature room at 21°C and shaken at 200 rpm for 3 h. Bioluminescence was measured using an Oriel Detection System Model 7070 (Oriel, Stratford, CT) as described by Heitzer et al. (1998). After the final bioluminescence measurement, an 0.5 mL aliquot of culture was removed and the final optical density (546 nm) was measured, converted to milligrams of protein based on a standard curve, and used to calculate the specific bioluminescence (namp/mg protein) by dividing the sample bioluminescence by total protein. The remaining culture was used to determine the initial toluene degradation rate.

Measurement of Tod Activity

Initial toluene degradation rates were used as an indicator of Tod activity in TVA8. To determine the initial degradation rates, 3.5 mL of the above culture were centrifuged, washed once with 4.0 mL MSM, centrifuged, resuspended in 4.0 mL MSM, and added to 25-mL glass vials with Teflon-lined caps. Toluene-saturated MSM was added to give an initial concentration of 6 mg toluene/L (liquid phase). Head-space toluene concentration was measured over time by removing 100- μ L samples from the vials and injecting the samples into a Hewlett Packard 5890 gas chromatograph equipped with a 30m Supelco VOCOL column, an electron capture detector (ECD), and a flame ionization detector (FID). The chromatography conditions were as follows: 50°C oven; 50°C ECD and FID; carrier gas flow rate was 6.8 mL helium/min; make-up gas flow rate was 49.6 mL nitrogen/min. The toluene degradation rate was determined by the change in head-space toluene concentration after 50 min and then normalized by the total protein.

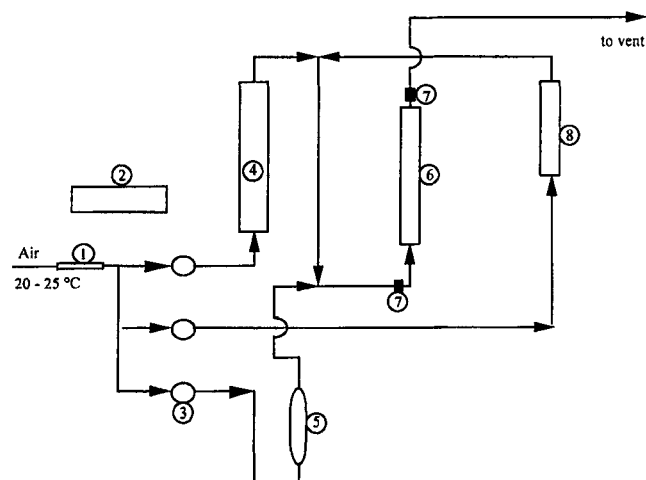
Reactor System Description

Figure 2 is a schematic representation of the reactor system. A Matheson Model 8284 Multiple Dyna-Blender (mass flow controller) and three Model 8272-0412 transducers controlled the air flow rates to all vessels. Vapor-phase TCE and toluene concentrations were measured over time by removing 100- μ L samples from sample ports and injecting the samples into a Hewlett Packard 5890 gas chromatograph as described above.

The RFB had the following properties during the experiments for TCE cometabolism: V_B was 483 mL, V_S was 332 mL, ϵ was 0.0015, d_s was 0.025 cm, δ was 0.0017 cm, and X_0 was 0.5 mg cells/mL. The biofilm thickness, δ , was calculated by assuming the sand particles and biofilm were spherical and by determining the difference in the radius for each, based on V_S and V_B , respectively.

Biofilm Growth

The biofilm growth medium was Lurea Broth with kanamycin (LBkan25) for selective pressure, and the



- | | |
|-------------------------|---------------------------|
| 1. Air Filter | 5. TCE/water Column |
| 2. Mass Flow Controller | 6. Radial Flow Bioreactor |
| 3. Mass Flow Transducer | 7. Sample Port |
| 4. Air Humidifier | 8. Toluene Column |

Figure 2. Schematic presentation of vapor-phase toluene/TCE biological degradation process using the RFB.

biofilm support was Sigma +70/-50 mesh sand. The composition of the LBkan25 medium was 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 25 μ g/mL kanamycin. Initially, a 100-mL starter culture was grown to an optical density of 1.1 (546 nm) in growth medium. Meanwhile, four 1-L Erlenmeyer flasks were filled with 250-g sand and 250-mL growth medium. Each flask was inoculated with 250 μ L of the starter culture. The flasks were shaken at 250 rpm, and sand and liquid phase samples were taken at random time points. The maximum number of colony forming units per gram of sand was found somewhere between 45 and 120 h after inoculation of the growth flasks, and the harvest time was 50 h (Shingleton, 1998).

Reactor Preparation

The sand from the growth flasks was harvested by discarding the residual LBkan50 liquid and adding 250 mL of fresh MSM. Nutrients were supplied by adding 1-g/L nutrient beads (24.1% N, 5.5% P, 0.7% S, 0.5% K, 3.1% micronutrients, w+/w+ to the slurry. The nutrient beads (Grace-Sierra Horticultural Products, Milpitas, CA) were designed for controlled release of nutrients upon exposure to moisture. The slurry was then poured into the annular region of the reactor. The MSM solution drained through the porous metal, while the sand and immobilized bacteria remained in the reactor.

MATHEMATICAL MODEL

Rouhana and colleagues (1997) developed the first mathematical model for the packed-bed RFB (Fig. 1)

based on the vapor-phase degradation of *p*-xylene. Conventional packed-bed, axial-flow bioreactors often have mass transfer, irregular flow, and ensuing scale-up problems. Relatively high surface area per volume ratios that increase mass transfer rates are possible in the RFB. Because of the relatively small bed length, the pressure drop across the RFB is low, and the feed components maintain constant physical properties. Uniform distribution of the feed stream along the porous-metal inner annulus reduces dry spot formation and channeling within the bed. Hence, compared to conventional axial-flow configurations, more ideal flow conditions and higher mass transfer rates in the radial-flow configuration facilitate the development of a more reliable mathematical model for prediction and scale-up.

The model proposed by Rouhana and colleagues (1997) assumed the bulk gas and biofilm had the same composition at any given point in the reactor. This current study on TCE cometabolism is the second RFB study. To formulate a more rigorous mathematical model, mass balances were performed on a differential section of the RFB to describe the radial transport of vapor-phase toluene and TCE through a bulk gas phase and the concomitant biological reaction in a stationary biofilm phase (Bird et al., 1960). Figure 3 presents a schematic diagram of the sand particles, the stationary biofilm, the theoretical liquid and gas films, and a differential section of the bulk gas phase. The biofilm phase is modeled as distributed stationary biomass sources and reactant sinks, where the mass transport rate to the biocatalyst is negligible relative to the intrinsic reaction rate. If the biofilm is considered a thin film, diffusion of substrate through the liquid film and the biofilm phase does not affect the observed reaction rate. To test the validity of the thin-biofilm assumption for toluene degradation in the RFB, Eq. (1) gives characteristic times for the reaction process (t_R) and for the diffusion process (t_M) (Characklis and Marshall, 1990),

$$\frac{t_R}{t_M} = \frac{-C_{f1}/(r_1 X)}{\delta^2/D_{L1}} = 130 \quad (1)$$

where $C_{f1} = 0.0026$ mg toluene/mL, $r_1 = 0.0097$ mg toluene/mg cell per min, $X = 0.5$ mg cell/mL, $\delta = 0.0017$ cm, and $D_{L1} = 7 \times 10^{-4}$ cm²/min for the conditions studied. The estimate indicates that t_M is 130 times less than the t_R in the biofilm. Therefore, diffusion of substrate into the theoretical liquid film and the biofilm is relatively fast and does not affect the observed reaction rate. As shown in Fig. 3, the biofilm concentration (C_{fi}) is then equal to the interfacial concentration of theoretical liquid film (C_{li}) which is at equilibrium with the interfacial concentration of the theoretical gas film (C_{gi}).

In the bulk gas phase, there is both a convective and a diffusive flux of toluene and TCE past the theoretical gas

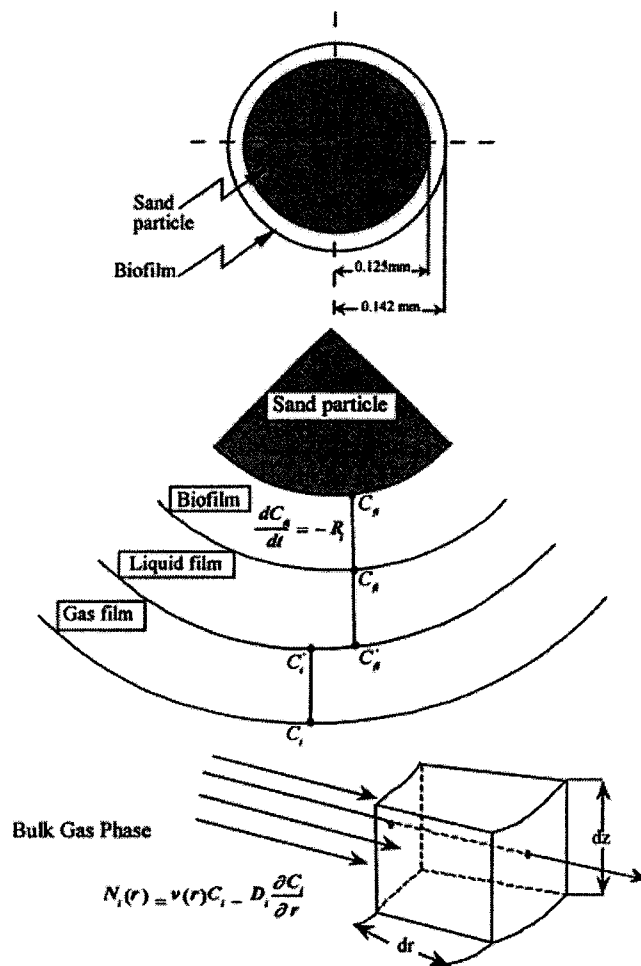


Figure 3. Schematic presentation of the biofilm immobilized on a sand particle and of a differential element of the RFB in radial coordinates. The theoretical gas and liquid films are shown (not to scale) with the interfacial gas (C_{gi}) and liquid (C_{li}) phase concentrations. The assumed concentration profiles through the gas (C_g) and liquid (C_l) films are shown. The concentrations in the gas and liquid films are related by Henry's law.

film. If diffusional limitations existed in the gas film, an increase in agitation, or flow rate, should increase the observed reaction rate. To test whether diffusional resistances in the gas film were significant, toluene degradation rates were measured for flow rates between 5.7 and 23 mL/min and were found to be independent of the air flow rate. Based on this observation, diffusion through the gas phase does not limit the reaction (Shingleton, 1998). Hence, in Fig. 3 the theoretical gas film concentration (C_{gi}) is identical to the bulk gas concentration (C_g).

Ultimately, equilibrium between the gas and the liquid phases occurs fast enough to assume that the biofilm concentration (C_{fi}) is related to the bulk gas concentration (C_g) phase through Henry's law. Chang and Alvarez-Cohen (1995) determined the Henry's law coefficients for toluene and TCE to be 0.27 and 0.36 (L water/L air), respectively.

The assumptions for the newly proposed RFB model are: negligible mass transfer resistance, stationary biomass phase with same properties as liquid water, no oxygen or nutrient limitations, toluene as the sole carbon source, constant concentrations in axial direction, negligible pressure drop, isothermal system, constant physical properties, and no channeling. Based on these assumptions, a differential mass balance written for the RFB is,

$$\frac{\partial C_i^*}{\partial t} - \frac{\tau D_i}{\Delta r^2} \frac{1}{r^*} \frac{\partial}{\partial r^*} \left(r^* \frac{\partial C_i^*}{\partial r^*} \right) - \frac{\tau r_1 u_1}{\Delta r^2} \left(\frac{1}{r^*} \right) \frac{\partial C_i^*}{\partial r^*} - \xi B_i F_i X^* C_i^* = 0 \quad \{i = 1, 2\} \quad (2)$$

where

$$C_i^* = \frac{C_i}{C_{i0}}, X^* = \frac{X}{X_0}, r^* = \frac{r_1}{\Delta r}, t^* = \frac{t}{\tau}$$

are the normalized variables. Model formulation details are given by Shingleton (1998).

The kinetic expressions for toluene ($i = 1$) and TCE ($i = 2$) cometabolism in Eq. (2) are based on Michaelis-Menten competitive-inhibition kinetics (Bailey and Ollis, 1986). The nondimensional parameter grouping B_i consists of the physical and rate constants for each compound. They are essentially nondimensional degradation rate constants for toluene and TCE, respectively, and are given as,

$$B_i = \left(\frac{1 - \varepsilon - \varepsilon_s}{\varepsilon} \right) \frac{\tau k_i X_0}{C_{i0}} \quad (3)$$

The nondimensional reaction rate function (F_i) is identified as,

$$F_i = \frac{1}{C_i^* + \frac{H_i K_i}{C_{i0}} \left(1 + \frac{C_{j0} C_j^*}{H_j K_j} \right)} \quad (4)$$

where $j \neq i = 1, 2$.

Equation (2) also contains the Tod activity parameter in the reaction term to account for differential Tod activity at low toluene concentrations. The correlation between the Tod activity parameter and toluene (inducer) concentration is developed in the next section. The boundary and initial conditions for Eq. (2) are,

$$C_i^*(0, r) = 0 \quad \{i = 1, 2\} \quad (5)$$

$$C_i^*(r_1) = 1 \quad \{i = 1, 2\} \quad (6)$$

$$\left. \frac{\partial C_i^*}{\partial r} \right|_{r=r_2} = 0 \quad \{i = 1, 2\} \quad (7)$$

The kinetic and cell growth parameters used in the proposed model are shown in Table I. The Michaelis-Menten parameters are those found by Kelly and colleagues (2000) for *P. putida* TVA8 in a chemostat fed with 80% strength MSM, toluene, TCE, and air.

The biomass growth equation is written as a modified form of an equation proposed by Criddle (1993),

Table I. Michaelis-Menten and biomass growth parameters.

Parameter	Value	Units	Reference
k_1	0.009722	mg toluene/mg cells per min	(Kelly et al., 2000)
k_2	0.000903	mg TCE/mg cells per min	(Kelly et al., 2000)
K_1	0.0027	mg toluene/mL	(Kelly et al., 2000)
K_2	0.0064	mg TCE/mL	(Kelly et al., 2000)
Y	1.0	mg cells/mg toluene	(Kelly et al., 2000)
T_c	0.0047	mg TCE/mg cells	(Yu, 1998)

$$\frac{dX^*}{dt^*} + (GRWTH)\xi F_1 C_1^* X^* + (TC)\xi F_2 C_2^* X^* + bX^* = 0 \quad (8)$$

where $(GRWTH) = -\tau Y k_1$ and $TC = (1/T_c)\tau k_2$ are dimensionless parameter groupings. The first term in Eq. (8) accounts for biomass growth upon metabolism of toluene, the primary growth substrate. The second term accounts for the inactivation of biomass due to the TCE-induced enzyme deactivation. The third term accounts for the loss of viable biomass due to cell maintenance requirements and is assumed to be negligible in this system.* The initial condition for Eq. (8) is,

$$X^*(0, r) = 1 \quad (9)$$

The growth parameters are those given by Yu (1998) for *P. putida* F1 in batch culture.

The development of the solution process necessitates grouping the kinetic expression into a nondimensional parameter group B_i and a specific reaction rate function F_i . The nonlinearity appearing in the reaction term in Eq. (2) requires a numerical technique capable of stabilizing the solution process. The nonlinearity appears when the normalized substrate concentration, C_i^* , is multiplied by both the normalized biomass concentration, X^* , and the Tod activity parameter, ξ . Because toluene is the growth substrate, the rate of biomass production is a function of toluene concentration. Because the activity parameter is a piecewise continuous function of toluene concentration, it also adds nonlinear characteristics to the model equations. The finite element method with first-order basis functions was used to solve the coupled equations. The Galerkin weak-state-ment (GWS) formulation was used to formulate the optimum solution (Baker and Pepper, 1991).

TOD ACTIVITY PARAMETER

For enzyme-catalyzed reactions, an expression for the maximum specific rate constant is given by Shuler and Kargi (1992) as,

$$k_2 = E_0 k \quad (10)$$

*Hence the constant, b becomes 0.

where k_2 is the maximum specific rate constant, k is the intrinsic reaction rate constant for the formation of the enzyme-substrate complex, and E_0 is the total enzyme concentration. In Eq. (10), k_2 increases only if the enzyme concentration increases. However, this relationship as written assumes a constant enzyme concentration that is not regulated by the inducer concentration. If the enzyme is inducible, the enzyme concentration is naturally a function of inducer concentration. Therefore, some correlation between enzyme (Tod) concentration or activity and inducer concentration (toluene) is required to provide full functionality in the reaction kinetics.

In previous TCE degradation studies with Tod-mediated cometabolism, the toluene concentrations were typically above 10 mg/L, where the bioluminescence becomes independent of inducer concentration (Applegate et al., 1998; Kelly et al., 2000). For toluene concentrations between 0 and 30 mg toluene/L, Fig. 4 presents the observed linear relationship ($R^2 = 0.9690$) between specific bioluminescence in the growing cell assays and the subsequent initial toluene degradation rate for each toluene concentration. Initial toluene degradation rates were used as a measure of Tod activity, which is a measure of effective Tod concentration. As the toluene concentration was increased from 0.1 to 30 mg toluene/L, specific bioluminescence increased approximately fivefold and the toluene degradation rate increased over fourfold. Figure 5 gives the correlation between normalized toluene concentration and normalized specific bioluminescence. The toluene concen-

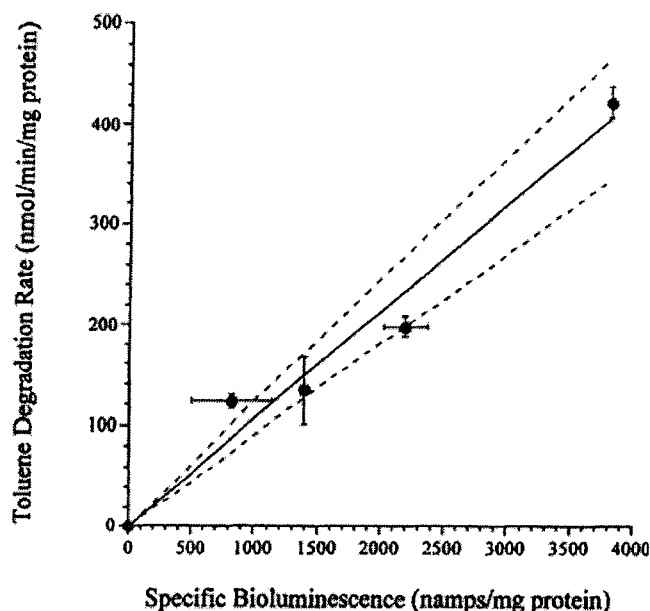


Figure 4. Correlation between initial toluene degradation rate in resting cell assays and the specific bioluminescence corresponding to toluene concentrations of 0, 0.1, 1, 10 and 30 mg toluene/L in the growing cell assays (slope = 0.11, $R^2 = 0.9852$). Ninety-five percent confidence interval for the regression is shown as dashed lines.

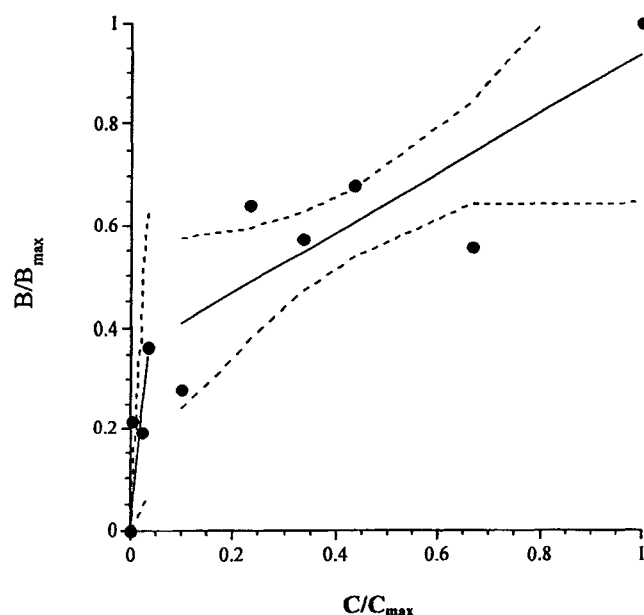


Figure 5. Correlation between specific bioluminescence ($B_{\max} = 3811$ namps/mg protein) after 3 h in the growing cell assays and normalized toluene concentration ($C_{\max} = 30$ mg toluene/L). Ninety-five percent confidence interval for the regression is also shown as dashed lines.

tration is normalized by C_{\max} (30 mg toluene/L), and the specific bioluminescence is normalized by B_{\max} (3,811 namps/mg protein). The data points from the growing-cell assay correlate the bioluminescent response to Tod activity after 3 h when *todC1* was fully induced (Shingleton et al., 1998).

Figures 4 and 5 form the basis of using the bioluminescent response of *P. putida* TVA8 as a tool to measure the Tod activity in the *P. putida* TVA8 cells. Because the specific bioluminescence and enzyme activity relationship is a linear relationship that passes through zero, the Tod activity can be found by multiplying the specific bioluminescence by 0.11. The relationship of Tod activity and specific bioluminescence is normalized as,

$$\frac{B}{B_{\max}} = \frac{R}{R_{\max}} = \xi \quad (11)$$

where B is the specific bioluminescence (namps/mg protein), R is the initial toluene degradation rate (nmol/min per mg protein), B_{\max} is the maximum specific bioluminescence, and R_{\max} is the maximum initial toluene degradation rate. The maximum Tod activity ($\xi = 1.0$) occurs at 30 mg toluene/L, which is supported by the saturational form shown by Applegate and colleagues (1998).

Based on a regression analysis with 95% confidence intervals as shown in Fig. 5, the Tod activity induced in the biofilm for concentrations of C_{fl} between 0 and 0.99 mg/L is written as,

$$\xi = d1 \frac{C_{\text{fl}}}{C_{\max}} = d1 \frac{C_{10} C_1^*}{H_1 C_{\max}} \quad 0 \leq \frac{C_{\text{fl}}}{C_{\max}} \leq 0.033 \quad (12)$$

and the Tod activity for C_{fl} greater than 0.99 mg/L is given as,

$$\xi = d2 \frac{C_{fl}}{C_{max}} + d3 = d2 \frac{C_{10} C_1^*}{H_1 C_{max}} + d3$$

$$0.033 < \frac{C_{fl}}{C_{max}} < 1.0 \quad (13)$$

where C_{max} is 30 mg/L and the regression coefficients are $d1$ (10.5), $d2$ (0.591), and $d3$ (0.349). A piecewise linear representation of the data was required to stabilize the numerical solution. The dividing point of 0.99 mg/L was chosen because the Tod enzyme concentration appears to be less dependent on toluene concentration at toluene concentrations above 1 mg/L, and 0.99 mg/L was the nearest experimental data point.

RESULTS

Abiotic Experiments

Under the conditions given in "Materials and Methods", the effects of adsorption and absorption in the RFB are negligible after the first 5 h of operation for flow rates of 8.5 mL/min, corresponding to a residence time of 0.09 min (data not shown). After 5 h, the loss of TCE and toluene through the reactor can be attributed to degradation by *P. putida* TVA8 (Shingleton, 1998).

Cometabolism

Experimental TCE cometabolism results are shown in Fig. 6, and experimental toluene degradation results are shown in Fig. 7. The initial biomass concentration was 0.5 mg/mL in the biofilm phase. Both figures are partitioned into three sections (a, b, and c), each corresponding to different feed conditions. Between 0 and 55 h, the TCE conversion is 10% and the toluene conversion is near 100%. The time between 55 and 80 h is a transient period where the toluene concentration increases from 0.33 to 0.61 mg/L and the TCE concentration decreases from 0.31 to 0.20 mg/L. As shown in Fig. 6, a shift in feed concentration has no significant effect on the TCE conversion, but the toluene conversion drops significantly from 99+ % to 90%. For these conditions, the predicted toluene conversion is approximately 97.5% and 95%, respectively. On average for the conditions given in Table II, the experimental TCE degradation rate is 0.28 $\mu\text{g}/\text{min}$ and the average predicted TCE degradation rate is 0.24 $\mu\text{g}/\text{min}$.

Influence of the Tod Activity Parameter on Model Predictions

Figures 8 and 9 show the effect of the enzyme activity on the model prediction of TCE, toluene, and biomass concentration profiles inside the RFB based on the following operating conditions: $C_{10} = 0.7$ mg/L, $C_{20} = 0.3$

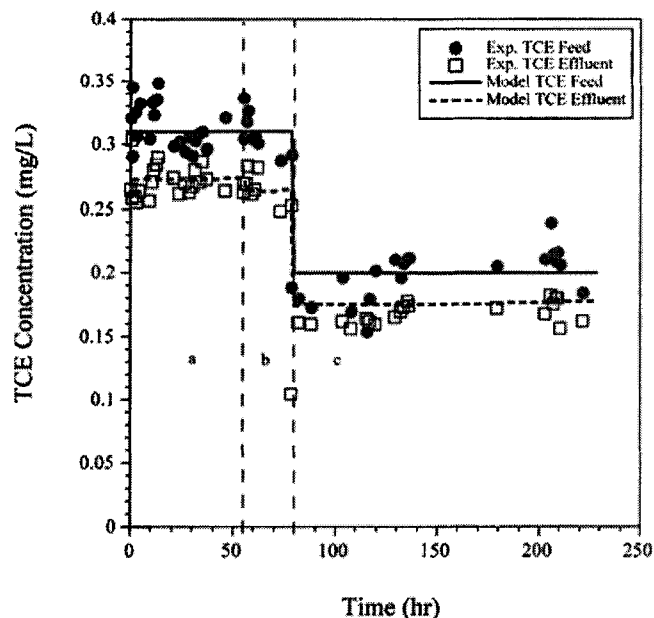


Figure 6. Experimental TCE degradation for various toluene and TCE feed concentrations (mg/L): (a) is 0.3 toluene and 0.3 TCE; (b) is 0.7 toluene and 0.2 TCE; (c) is 0.6 toluene and 0.2 TCE. $C_{10} = 0.7$ mg/L, $C_{20} = 0.3$ mg/L, $X_0 = 0.5$ mg cells/mL, and $V = 6.5$ mL/min.

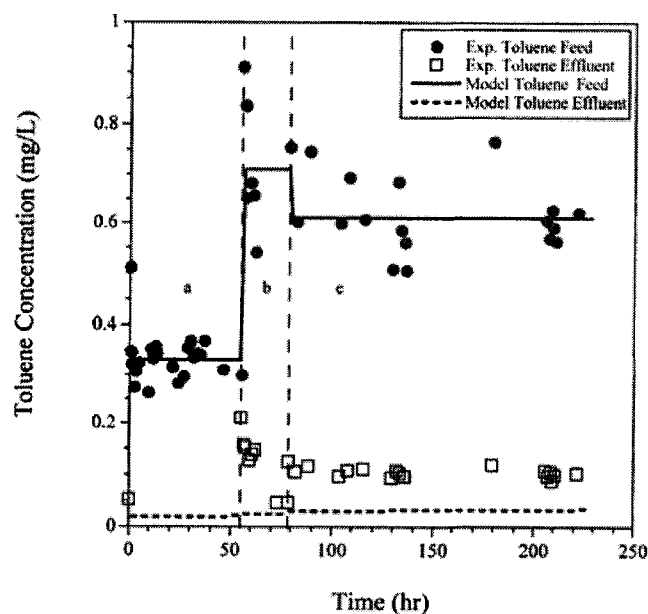


Figure 7. Experimental toluene degradation for various toluene and TCE feed concentrations (mg/L): (a) is 0.3 toluene and 0.3 TCE; (b) is 0.7 toluene and 0.2 TCE; (c) is 0.6 toluene and 0.2 TCE. $C_{10} = 0.7$ mg/L, $C_{20} = 0.3$ mg/L, $X_0 = 0.5$ mg cells/mL, and $V = 6.5$ mL/min.

mg/L, $X_0 = 0.5$ mg cells/mL, and $V = 6.5$ mL/min ($\tau = 0.1$ min). These were the conditions chosen to investigate the effects of the Tod activity correlation on the model predictions. Figure 8 presents the model prediction without the Tod activity parameter, and Fig. 9 presents the model prediction with the Tod activity parameter

Table II. Experimental and predicted TCE degradation rates for the various concentrations of toluene and TCE in the feed stream and for a volumetric feed rate of 6.5 mL/min ($\tau = 0.1$ min).

Case	Toluene (mg/L)	TCE (mg/L)	Experimental TCE degradation rate ($\mu\text{g}/\text{min}$)	Predicted TCE degradation rate ($\mu\text{g}/\text{min}$)
1	0.33	0.31	0.26 ± 0.2	0.22
2	0.71	0.31	0.28 ± 0.2	0.29
3	0.61	0.20	0.29 ± 0.2	0.22

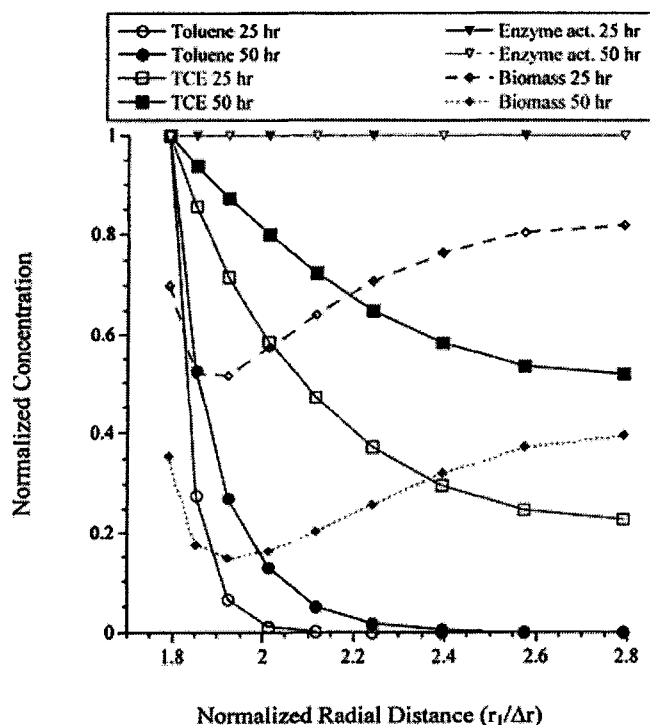


Figure 8. Predicted concentration profiles for toluene, TCE, enzyme activity, and biomass as a function of the normalized radial distance in the RFB where ξ is assumed to be 1.0 and constant. Symbols correspond to the location of nodes in the finite element mesh. $C_{10} = 0.7$ mg/L, $C_{20} = 0.3$ mg/L, $X_0 = 0.5$ mg cells/mL, and $V = 6.5$ mL/min.

added to the Michaelis-Menten kinetic expression. In these figures, the concentrations are normalized and are plotted as a function of the normalized radial distance ($r_1/\Delta r$) in the RFB. The predicted effluent concentration can be found at the last point in the reactor where $r_1/\Delta r$ is 2.79. According to both Fig. 8 and Fig. 9, the predicted biomass concentration initially decreases in the front of the reactor where TCE degradation rate and the resulting toxicity are high. The biomass concentration then increases as both the TCE degradation rate and the resulting toxicity decrease. The spatial biomass distribution also changes as a function of run time. Figure 8 shows the concentration profiles where the Tod activity is not a function of toluene concentration, that is, ξ is 1.0 and constant. This prediction shows that biomass concentration decreases by approximately 50% to 70% throughout the length of the bed. During this time in-

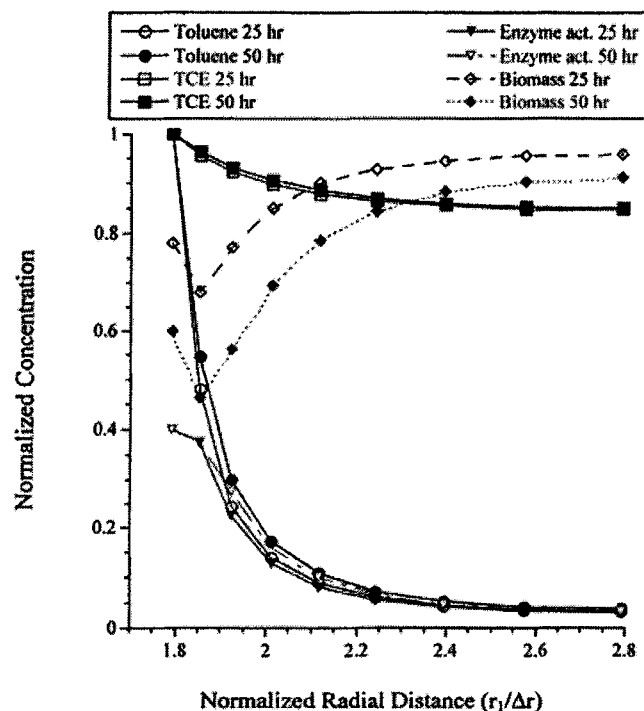


Figure 9. Predicted concentration profiles for toluene, TCE, enzyme activity, and biomass as a function of the normalized radial distance in the RFB where ξ is a function of toluene concentration (C_1). Symbols correspond to the location of nodes in the finite element mesh. $C_{10} = 0.7$ mg/L, $C_{20} = 0.3$ mg/L, $X_0 = 0.5$ mg cells/mL, and $V = 6.5$ mL/min.

terval, the effluent TCE concentration increases, and the predicted TCE conversion decreases from 75% to 45%, which is fivefold to threefold higher than the experimental conversion, respectively.

Figure 9 shows the radial concentration profiles where Tod activity is a function of toluene concentration, as shown in Eq. (12) and Eq. (13). Between 25 and 50 h, the predicted biomass profiles change only 25% at the front of the reactor and only 10% at the exit point. Both the TCE and toluene concentration profiles are relatively constant during this period, indicating steady-state degradation of each compound. The predicted effluent toluene and TCE concentration in Fig. 9 also matches both the experimental and predicted effluent concentration in Fig. 6 and Fig. 7.

Figure 10 shows that the Tod activity parameter had a large qualitative and quantitative impact on the predicted TCE effluent concentration over time in the RFB. The experimental data in Fig. 6 show the effluent TCE concentration to be relatively constant over time. However, in Fig. 10, where ξ is 1.0 and independent of toluene concentration, the predicted TCE conversion varies with time from 80% after 10 h to 20% after 68 h, which is neither qualitatively nor quantitatively consistent with the experimental data. When the Tod activity was a function of toluene concentration, the predicted TCE concentration in the effluent was constant at 0.26

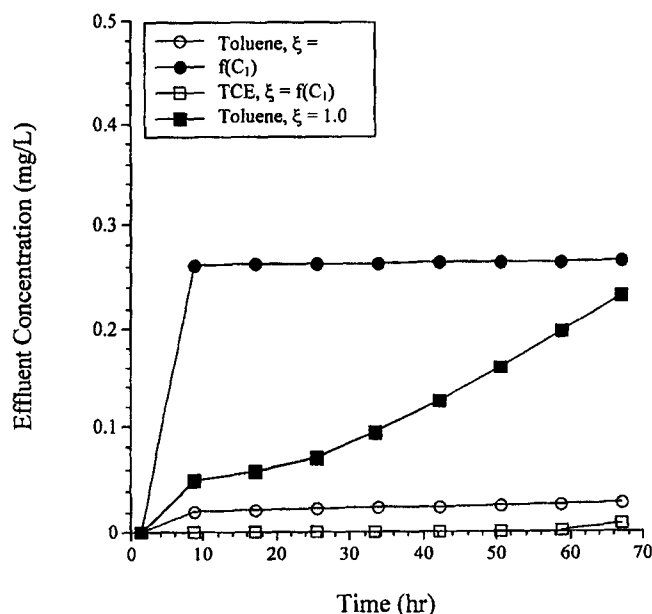


Figure 10. Effect of the *Tod* activity parameter on the time-dependent model predictions for effluent TCE and toluene concentrations when ξ is a function of toluene concentration (circles) and ξ is 1.0 (squares). $C_{10} = 0.7$ mg/L, $C_{20} = 0.3$ mg/L, $X_0 = 0.5$ mg cells/mL, and $V = 6.5$ mL/min.

mg/L and the predicted conversion was 13%, which was both qualitatively and quantitatively consistent with the experimental data.

DISCUSSION

P. putida TVA8 has both a bioluminescent response (*tod-lux* fusion) and a *Tod* activity (wild-type *tod* operon) that are dependent on toluene concentration, especially at concentrations less than 5 mg/L. From experimental data, a new correlation was found between toluene concentration and induced *Tod* activity as measured by a bioluminescent response of *P. putida* TVA8. The correlation was presented as a nondimensional *Tod* activity parameter for use in the Michaelis-Menten competitive-inhibition kinetic expression to account for differential *Tod* induction at low toluene concentrations.

For toluene concentrations of 0.3 to 0.7 mg/L and a 0.1-min residence time, the steady-state TCE degradation rate in the RFB was 0.28 μ g/min. For at least 220 h, relatively low toluene concentrations less than 1 mg/L in the feed stream were sufficient to maintain cell viability and enzyme activity, while reducing the competitive-inhibition effects to a point where measurable TCE cometabolism was possible. However, the model results in Fig. 9 indicate that the full capacity of the RFB is not utilized. The TCE degradation rate slows dramatically in the middle of the bed. When the toluene concentration drops below 0.05 mg/L, the *Tod* activity is too low for significant TCE degradation. Therefore, in future

studies the toluene concentration in the feed stream should be increased by approximately 1.5- to 2-fold to optimize the performance of the RFB.

When the *Tod* activity was assumed to be maximally induced ($\xi = 1.0$) and independent of toluene concentration, major qualitative and quantitative deviations from experimental data existed. First, as shown in Fig. 10, the predicted TCE conversion was significantly higher than the experimental conversion. The high predicted degradation was caused by a lack of functionality between toluene concentration and *Tod* activity. Without the proper correlation, the model predicted maximal *Tod* activity in the RFB where both low toluene concentrations and low competitive inhibition prevailed. Second, the predicted TCE degradation rate in the RFB also decreased over time, whereas the experimental degradation rate was constant over time. Both the high toxicity associated with a high predicted TCE degradation rate and the low toluene concentrations in the second half of the bed decreased the biomass concentration by 50% to 70% between 25 and 50 h, as shown in Fig. 8. Therefore, when *Tod* activity is assumed to be maximally induced in the Michaelis-Menten kinetics expression under conditions of low steady-state feed concentrations of toluene, the predicted TCE degradation rate is erroneously high and decreases over time due to erroneously high cellular toxicity and low biomass growth.

Mathematical functionality between inducer concentration and enzyme activity naturally exists, but such functionality is difficult to measure, especially *in vivo*. Therefore, the *tod-lux* bioreporter *P. putida* TVA8 developed by Applegate and colleagues (1998) was an essential biological tool for measuring the correlation between induced *Tod* activity and toluene concentration. The development of the *Tod* activity parameter provided fundamental data on modeling *Tod* induction by toluene, and ultimately enabled qualitatively and quantitatively accurate predictions of the experimental data for steady-state, *Tod*-mediated TCE cometabolism.

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NOMENCLATURE

- b cell decay constant
- B specific bioluminescence (namps)
- B_i nondimensional reaction rate constant of component i
- B_{\max} maximum specific bioluminescence (namps)
- C_i concentration of toluene (1) or TCE (2) in the bulk gas phase (mg/L)
- C_{i0} concentration of component i in the bulk gas phase at r_1 (mg/L)
- C_{fi} concentration of component i in the biofilm phase (mg/L)
- C_{\max} toluene concentration for maximal *Tod* induction (mg/L)
- d_s sand diameter (cm)

D_i	effective dispersion coefficient for component i in air (cm^2/s)
F_i	nondimensional function in kinetic expression for component i
H_i	Henry's law constant for component i (L water/L air)
k_i	maximum specific degradation rate for component i (mg i /mg cells per min)
K_i	Michaelis-Menten half-saturation constant for component i (mg i /L)
r_1	radius of reactor inlet (inner annulus) (cm)
r_2	radius of reactor outlet (outer annulus) (cm)
Δr	radial distance of the packed bed (cm)
t_M	characteristic diffusion time (min)
t_R	characteristic reaction time (min)
T_c	transformation capacity (mg TCE/mg cells)
V_B	bed volume (mL)
V_S	sand volume (mL)
X	cell mass concentration (mg cells/L)
X_0	initial cell mass concentration (mg cells/L)
Y	yield coefficient (mg cells/mg toluene)
δ	biofilm thickness (cm)
ε	void fraction (cm^3 gas/ cm^3 bed)
ε_s	solid fraction (cm^3 solid/ cm^3 bed)
τ	residence time (min)
ξ	nondimensional Tod activity parameter

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Controlled Field Release of a Bioluminescent Genetically Engineered Microorganism for Bioremediation Process Monitoring and Control

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Pseudomonas fluorescens HK44 represents the first genetically engineered microorganism approved for field testing in the United States for bioremediation purposes. Strain HK44 harbors an introduced *lux* gene fused within a naphthalene degradative pathway, thereby allowing this recombinant microbe to bioluminesce as it degrades specific polycyclic aromatic hydrocarbons such as naphthalene. The bioremediation process can therefore be monitored by the detection of light. *P. fluorescens* HK44 was inoculated into the vadose zone of intermediate-scale, semicontained soil lysimeters contaminated with naphthalene, anthracene, and phenanthrene, and the population dynamics were followed over an approximate 2-year period in order to assess the long-term efficacy of using strain HK44 for monitoring and controlling bioremediation processes. Results showed that *P. fluorescens* HK44 was capable of surviving initial inoculation into both hydrocarbon contaminated and uncontaminated soils and was recoverable from these soils 660 days post inoculation. It was also demonstrated that strain HK44 was capable of generating bioluminescence in response to soil hydrocarbon bioavailability. Bioluminescence approaching 166 000 counts/s was detected in fiber optic-based biosensor devices responding to volatile polycyclic aromatic hydrocarbons, while a portable photomultiplier module detected bioluminescence at an average of 4300 counts/s directly from soil-borne HK44 cells within localized treatment areas. The utilization of *lux*-based bioreporter microorganisms therefore promises to be a viable option for in situ determination of environmental contaminant bioavailability and biodegradation process monitoring and control.

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Introduction

As part of the U.S. Department of Energy Natural and Accelerated Bioremediation Research program, investigations were initiated to advance applications of genetically engineered microorganisms (GEMs) for subsurface soil bioremediation. Part of the broad goals of this investigation was to achieve U.S. Environmental Protection Agency (U.S. EPA) permission for the first field release of engineered microorganisms for use in bioremediation and to develop a field facility for environmentally controllable and replicated field tests of GEMs. A third goal of the investigation was to release an engineered microorganism into the subsurface soil environment to test fundamental hypotheses on survivability of the organisms and their application in bioremediation process monitoring and control. The first of these broad goals was attained when the University of Tennessee in collaboration with Oak Ridge National Laboratory (ORNL) received in March of 1996 a consent order (Premanufacturing Notification #P95-1601) sanctioning the release of a GEM for applications in bioremediation process monitoring and control (1). The second goal was accomplished by redesign of a large soil lysimeter array at the ORNL Y-12 facility in Oak Ridge, TN for controlled research application of bioremediation in a reconstructed vadose zone (2). The final goal of this investigation is the object of this report and focuses on the application of the GEM *Pseudomonas fluorescens* HK44 for polycyclic aromatic hydrocarbon (PAH) bioremediation process research. Strain HK44 contains an introduced naphthalene catabolic plasmid (pUTK21) mutagenized by transposon insertion of *lux* (bioluminescent) genes (3). The original host microorganism and plasmid were isolated from manufactured gas plant (MGP) soils heavily contaminated by PAHs. Strain HK44 degrades a number of two-to-three ring PAHs as well as other substituted PAHs due to the naphthalene catabolic pathway conferred by plasmid pUTK21 (4). Strain HK44 additionally produces bioluminescent light in response to naphthalene, salicylate, or 4-methyl salicylate when physiologically active during a bioremediation process. The study was conducted in the vadose zone of a constructed subsurface soil profile in six replicated soil lysimeters with and without contaminant PAHs (2). Test initiation began on October 30, 1996 and continued for an approximate 2-year period thereafter. The objectives of this research were to fundamentally test whether the engineered HK44 strain could be successfully introduced and maintained in a soil environment and to evaluate the use of in situ bioluminescence as a monitoring variable for online control of bioremediation.

Experimental Section

The Lysimeter Facility. Six subsurface lysimeters were utilized for the field release experiments. Lysimeters were 4 m deep by 2.5 m in diameter constructed of epoxy-coated, galvanized steel pipe buried 3 m below ground surface. A seventh lysimeter was filled with groundwater and used to adjust water levels in the other lysimeters. Each lysimeter was covered with a stainless steel lid. Lysimeters were arranged around a 6 m deep by 6 m diameter central core containing computer equipment, monitoring devices, and associated plumbing. Design and operating details of the lysimeter facility are available (2).

Each lysimeter containing soil was filled with a stratified bed containing a 31 cm layer of graded gravel, followed by a 61 cm layer of coarse sand, a 92 cm layer of clean soil, a 92 cm layer designated as the treatment zone, and a 61 cm uncontaminated soil cap (Figure 1). The soil, a Huntington

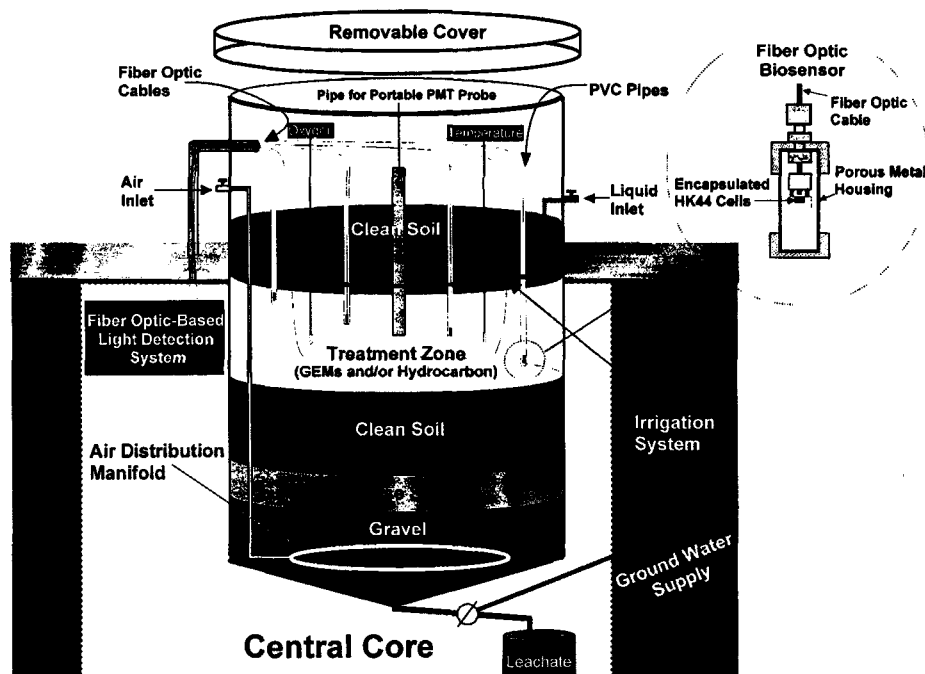


FIGURE 1. Schematic of a lysimeter illustrating the stratified soil layers and implementation of various instrumentation, including the fiber optic biosensor.

loam consisting of 42% sand, 40% silt, 18% clay, and 1.3% organic carbon, was loaded in approximate 10 cm increments, with each application packed to a dry density of 1300 kg/m³. The treatment layers received PAH-contaminated soil and/or an HK44 inoculum. A concrete mixing truck was used to mix and contaminate 23 m³ of soil with the PAH contaminants to produce a final concentration of 1000 mg/kg naphthalene, 100 mg/kg anthracene, and 100 mg/kg phenanthrene. Although naphthalene was the only PAH contaminant specific for strain HK44, the additional presence of anthracene and phenanthrene aided in creating a heterogeneous waste mixture more representative of a typical MGP site. Lysimeters 1, 2, and 4 received a 92 cm layer of PAH-contaminated soil inoculated with HK44, lysimeters 3 and 5 received uncontaminated soil inoculated with HK44, and lysimeter 6 received a layer of PAH-contaminated soil not inoculated with HK44.

To allow PAH sorption and soil weathering, the contaminated and uncontaminated soils were originally scheduled to be stored under plastic tarps for 100 days. However, due to delays in receiving final permission for HK44 release, the soils were subjected to an additional 160 days of storage, resulting in significant (>90%) desorption and volatile loss of the PAH contaminants. It was therefore necessary to augment the contaminants. Consequently, on day 135 following test initiation, lysimeters 1, 2, 4, and 6 received supplementary contaminants consisting of 24 kg naphthalene and 2.4 kg anthracene dissolved in 833 L Exxon Univolt 60 transformer oil. This oil was chosen because it was representative of an environmental contaminant, and it met requirements that it not contain hazardous constituents as listed by the Resource Conservation and Recovery Act. Each of lysimeters 1, 2, 4, and 6 received 208 L through irrigation tubing located directly above the treatment zone. Assuming uniform distribution of oil in the soil, this produced an approximate loading capacity of 1000 mg naphthalene/kg soil and 100 mg anthracene/kg soil throughout the treatment zone. The oil was added, and the water table rapidly lowered to produce a pressure drop that, along with gravity, facilitated the transport of oil into the contamination zone. Seven days later, 190 L of an inorganic nutrient solution (2 g NaNO₃, 0.75 g KH₂PO₄, 0.003 g FeCl₃, 0.1 g MgSO₄, 0.005 g CaCl₂, and 0.25

g Na₂HPO₄ per L of water) was also added to each of lysimeters 1, 2, 4, and 6 through irrigation tubing buried directly above the soil treatment zone (2). The nutrients and PAH-oil mixture were intended to restimulate HK44 population growth. After the addition of nutrients, air was pumped through a distribution manifold located at the bottom of lysimeters 1, 2, 4, and 6 at approximately 3.5 m³/h for a period of 2 h twice per week to aerate the soil (2).

Inoculation of Soil. The HK44 inoculum was prepared in a 500 L New Brunswick fermenter (2). After a 22 h growth period, the culture was harvested in a continuous centrifuge, and the resulting cell paste was resuspended in 227 L of saline and stored overnight at 4 °C. The next day, the saline resuspension was transported to the lysimeter site. Each 10 cm soil application within the 92 cm treatment zone was sprayed with approximately 4 L of resuspended cells using a backpack spray tank equipped with an extended nozzle (5).

Detection of Bioluminescence from *P. fluorescens* HK44.

A multiplex light detecting system using fiber optics and photon counting was used to monitor HK44 bioluminescence from biosensors and directly from the soil. Biosensors were deployed at depths of 105, 120, 138, and 152 cm within 4 cm diameter PVC pipes installed in lysimeters 1, 2, and 3 (four pipes per lysimeter, 12 total biosensors) to detect vapor phase naphthalene. The biosensors consisted of a subassembly that suspended alginate-encapsulated HK44 cells below a 1 mm diameter fiber optic cable, enclosed within a light-tight housing constructed from DuPont Delrin plastic and a porous stainless steel tube (Figure 1) (6, 7). Thirteen other fiber optic cables were buried directly in the soil at various depths within lysimeters 1, 2, and 3. Light emitted from HK44 cells was transferred through the fiber optic cables to a liquid light guide that was attached to a rotational stage mounted in a light-tight enclosure (8). The liquid light guide rotated to a position directly in front of each fiber optic probe and paused for the collection of light for 5 s (the recorded counts are an average of 50 counting events, each lasting 0.1 s). Photons collected by the light guide were detected with a photomultiplier tube (PMT) (Model R-4632, Hamamatsu, Middlesex, NJ) cooled to -35 °C. The pulses from the PMT were

processed by a photon counting module (Model C3866, Hamamatsu), and the data were stored in a computer database. The light guide continued this computer-controlled motion until light was collected from each fiber optic cable. After all fibers were analyzed, the computer-controlled rotational stage continuously repeated the cycle.

A portable PMT-based system was also constructed to monitor bioluminescence directly from soil-borne HK44 cells. This light detection system consisted of a light-tight housing containing a PMT module equipped with a microcontroller integrating all components necessary for photon counting (Model HC135-01, Hamamatsu). A laptop computer was used to set and control the operating parameters of the device and to receive and store digitized data. The probe was lowered into 7.6 cm diameter black ABS (acrylonitrile-butadiene-styrene) pipes that extended to various depths into the soil treatment zone. In most cases, clear Plexiglas windows were attached to the distal end of the buried pipes to minimize the transfer of oxygen to bacteria directly beneath the window. Localized supplements consisting of 100 mL of water and 300 mg of naphthalene, 100 mL of inorganic nutrient medium and 300 mg of naphthalene, or just 100 mL of inorganic nutrient medium were added to the soil prior to installation of the pipes as a means of stimulating HK44 populations.

Measurement of Environmental Parameters. The lysimeters were equipped with monitoring devices to measure soil oxygen concentrations and temperature (2). Oxygen sensors were placed in lysimeters 1, 2, 3, and 5 and a temperature sensor in lysimeter 2. Sensors were programmed to take readings every 30 min.

Soil Sampling. Soil cores, 1.8 m in length and 2.3 cm in diameter, were removed from each lysimeter using a Cole-Parmer soil sampler (Cole-Parmer Industries, Vernon Hills, IL). Cores traversed through the initial 61 cm of clean soil (which was subsequently discarded), into the 92 cm treatment zone, and finally into the 30 cm of soil directly beneath the treatment zone. Each core was then divided into four 30.5 cm sections, partitioning the treatment zone into three separate sections and producing a fourth section representing soil located directly beneath the treatment zone. Each section was thoroughly mixed and stored separately in sterile Whirlpak bags (Nasco, Ft. Atkinson, WI). A 5 g soil sample was immediately removed from each bag and heated to constant weight at 104 °C to determine moisture content.

Microbiological Analyses. One gram of soil samples was removed from each Whirlpak bag, suspended in 9 mL of 0.1% sodium pyrophosphate, and vortexed vigorously for 1 min to detach bacterial cells from soil particles. Dilutions were then prepared in saline and plated in triplicate on yeast extract-peptone-glucose (YEPG) agar plates to establish a general profile of heterotrophic microbial populations and on yeast extract-peptone-sodium salicylate (YEPSS) plates containing tetracycline at 14 mg/L to selectively obtain tetracycline resistant microbial populations inclusive of *P. fluorescens* HK44 (9). All plates contained cycloheximide at 14 mg/L to inhibit fungal growth. Plates were enumerated after 4 days incubation. Colonies from YEPSS plates containing between 30 and 300 colonies (i.e., those plates that were statistically countable) were periodically transferred to 82 mm diameter nylon membranes and hybridized with a 0.3 Kb ³²P-labeled *luxA* gene probe under high stringency conditions in order to verify HK44 genotype (10, 11). Colonies producing positive signals above background, as determined on a Molecular Dynamics Storm 840 Phosphorimager, were classified as *P. fluorescens* HK44.

Analysis of Soil Contaminants. PAHs in the soil were extracted from each sample using a solvent extraction procedure, and their individual concentrations were determined with a gas chromatograph-mass spectrometer (GC/

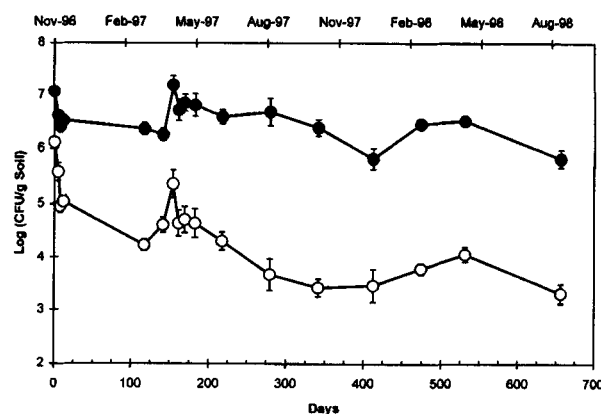


FIGURE 2. Relative heterotrophic (●) and presumptive *P. fluorescens* HK44 (○) population dynamics occurring in the PAH-contaminated lysimeter replicates (lysimeters 1, 2, and 4) during the field release. Error bars (not shown when smaller than symbol) represent standard error of the mean ($n = 6, 9, 18, 26, 35, 44$, or 59 depending on number of soil cores sampled).

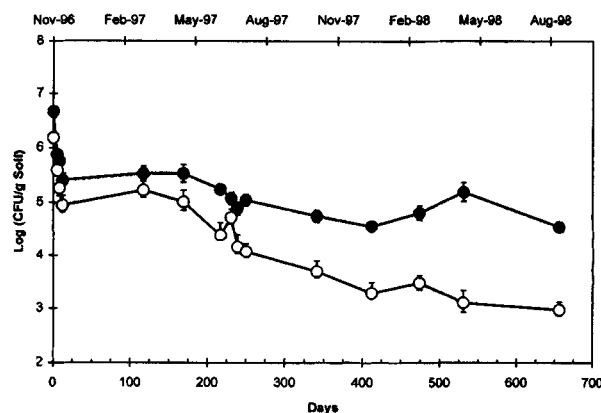


FIGURE 3. Relative heterotrophic (●) and presumptive *P. fluorescens* HK44 (○) population dynamics in the uncontaminated lysimeter replicates (lysimeters 3 and 5) over the duration of the field release. Error bars determined as for Figure 2, except $n = 6, 12, 18, 21$, or 30.

MS) (5973 Hewlett-Packard) using a modified EPA methodology (12), as described by Cox et al. (2).

Results

Six lysimeters were monitored over the duration of the study. Lysimeters 1, 2, and 4 were replicates representing HK44 inoculated/PAH-contaminated soils, lysimeters 3 and 5 were replicates representing HK44 inoculated/uncontaminated soils, and lysimeter 6 served as a control, containing noninoculated/PAH-contaminated soil. Data are presented as mean values (\pm standard error) for each replicate group, herein designated as PAH-contaminated (lysimeters 1, 2, and 4), uncontaminated (lysimeters 3 and 5), and control (lysimeter 6).

Survival and Persistence of *P. fluorescens* HK44. Upon initial inoculation, presumptive *P. fluorescens* HK44 concentrations as determined by selective plating on YEPSS plates were determined to be $1.5 (\pm 0.26) \times 10^6$ cfu/g soil in the PAH-contaminated lysimeters (Figure 2) and $1.7 (\pm 0.26) \times 10^6$ cfu/g soil in the uncontaminated lysimeters (Figure 3). Within 2 weeks, mean presumptive HK44 populations had decreased to $2.4 (\pm 0.68) \times 10^5$ cfu/g for the PAH-contaminated soil lysimeters and $3.4 (\pm 1.5) \times 10^5$ cfu/g for the uncontaminated soil lysimeters. Using the first-order decay model, $\ln N = \ln N_0 - (k_d \cdot t)$, where N_0 is the initial cell population, N is the cell population at time t , and k_d is the

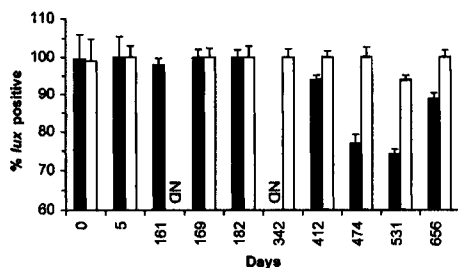


FIGURE 4. The percent of tetracycline resistant colonies growing on YEPSS plates that positively hybridized with the *luxA* gene probe in the PAH-contaminated lysimeter replicates (■) (error bars determined as for Figure 2) as compared to the uncontaminated lysimeter replicates (□) (error bars determined as for Figure 3). No *luxA*-positive hybridizations were detected in control lysimeter 6. ND = not determined.

first-order decay-rate constant (13), the rate of decay was determined to be -0.23 day^{-1} ($R^2 = 0.8435$) for the PAH-contaminated soils and -0.24 day^{-1} ($R^2 = 0.9915$) for the uncontaminated soils. Thus, the initial response to HK44 inoculation was similar for both contaminated and uncontaminated soils.

PAHs, transformer oil, and inorganic nutrients were added back to lysimeters 1, 2, and 4 135 days after initial HK44 introduction. Control lysimeter 6 received similar treatments. In the PAH-contaminated soils, this treatment resulted in a mean increase in presumptive HK44 numbers from $4.0 (\pm 2.1) \times 10^4 \text{ cfu/g soil}$ on day 117 to $3.7 (\pm 2.6) \times 10^5 \text{ cfu/g soil}$ on day 154 (growth rate = $+0.06 \text{ day}^{-1}$) (Figure 2). This growth rapidly depleted soil oxygen supplies, instigating the installation of a supplementary aeration system on day 160 (2). However, populations continued to decline.

Due to the presence of both *P. fluorescens* HK44 and indigenous tetracycline resistant microorganisms on YEPSS plates, it was required that hybridization assays be performed to genetically verify true HK44 isolates. A ^{32}P -labeled *luxA* gene probe was used for this purpose. The percentage of the tetracycline resistant population positively hybridizing to the *luxA* probe is shown in Figure 4. Prior to day 400, relatively few differences were seen between colony hybridization estimates and YEPSS enumerations in both PAH-contaminated and uncontaminated lysimeters. However, after day 400, YEPSS plate counts in the PAH-contaminated lysimeters generally exhibited a 10–25% higher estimate of HK44 concentrations than did the more stringent colony hybridization assessments, suggesting that HK44 populations were decreasing to a level where the background tetracycline population became detectable and more significant. Examination of colony morphology after day 400 also indicated that white colonies, as opposed to the yellowish HK44 colonies, were appearing on YEPSS plates. This colony morphology was also observed in tetracycline resistant colonies isolated from control lysimeter 6, which remained stable throughout the study (Figure 5) and continually exhibited a *luxA*⁺ genotype. This competing population did not substantially appear in the uncontaminated lysimeters, where essentially 100% of the colonies appearing on YEPSS plates were verified as *P. fluorescens* HK44 through *luxA* hybridization (Figure 4).

A complete overview of HK44 population dynamics as determined by colony hybridization is shown in Figure 6. In general, colony hybridization profiles tracked those of YEPSS plate counts, except after day 400 when the indigenous tetracycline resistant microorganisms began to predominate. For example, the rapid reduction in HK44 numbers immediately after soil inoculation was verified using colony hybridization data, as was the general HK44 population decline occurring thereafter. In the PAH-contaminated soils

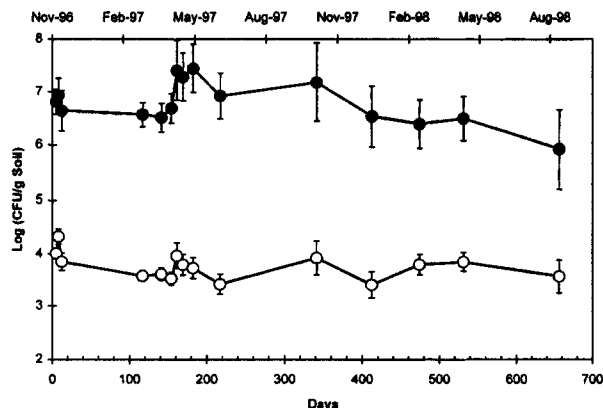


FIGURE 5. Relative heterotrophic microbial populations in control lysimeter 6 (●). An indigenous tetracycline resistant microbial population (○) was present throughout the study at an average concentration of $2.0 (\pm 0.74) \times 10^4 \text{ cfu/g soil}$. Error bars determined as for Figure 2, except $n = 6, 9, 12, 15$, or 26.

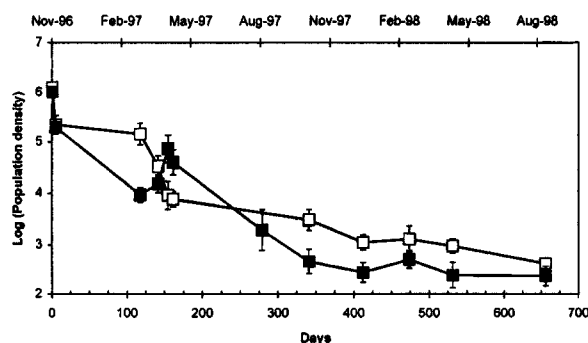


FIGURE 6. *P. fluorescens* HK44 population dynamics in the PAH-contaminated lysimeter replicates (□) (error bars determined as for Figure 2) and uncontaminated lysimeter replicates (■) (error bars determined as for Figure 3) as confirmed by colony hybridization.

this decline produced an order of magnitude difference between days 154 (mean HK44 cell count = $4.5 (\pm 1.7) \times 10^4 \text{ cfu/g soil}$) and 474 (mean HK44 cell count = $1.7 (\pm 1.3) \times 10^3 \text{ cfu/g soil}$) at a first-order rate constant of -0.017 day^{-1} ($R^2 = 0.8580$). A similar decay rate of -0.014 day^{-1} ($R^2 = 0.8956$) was observed in the uncontaminated lysimeters between days 117 (mean HK44 cell count = $3.6 (\pm 1.2) \times 10^5 \text{ cfu/g soil}$) and 474 (mean HK44 cell count = $2.2 (\pm 0.64) \times 10^3 \text{ cfu/g soil}$).

In addition to monitoring HK44 populations, general heterotrophic microbial populations were also determined using nonselective YEPG agar medium (Figures 2, 3, and 5). After the initial introduction of HK44 cells into the soil, a decrease in overall heterotrophic microbial populations was observed in all lysimeter treatments, with first-order exponential decay-rate constants of -0.11 day^{-1} ($R^2 = 0.7621$) for PAH-contaminated soils and -0.23 day^{-1} ($R^2 = 0.9451$) for uncontaminated soils. These decay rates were similar to those observed for HK44 populations. Heterotrophic microbial populations were also stimulated by the addition of the nutrient medium on day 135, and, as for HK44, quickly declined after this addition (Figures 2 and 5). Besides these noted exceptions, heterotrophic microbial populations capable of growth on YEPG plates generally remained stable with time.

Laboratory Analysis of Organic Contaminants. Initial GC/MS analysis of PAH-contaminated soils failed to detect quantifiable concentrations of naphthalene due to loss during extended storage of the soil prior to loading of the lysimeters. GC/MS analysis of soil cores taken within 1 month after the supplementary oil addition produced contaminant concen-

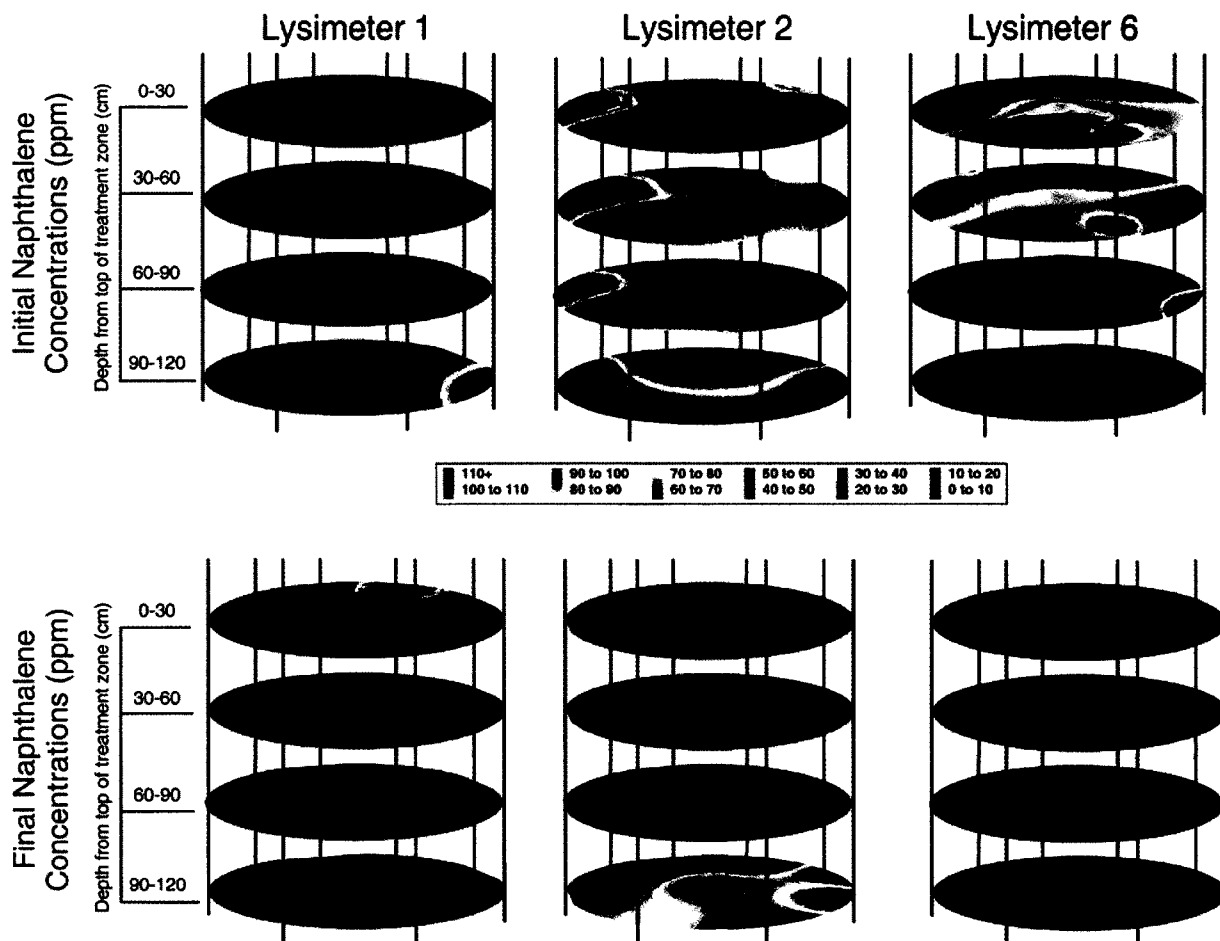


FIGURE 7. Initial and final naphthalene concentrations (ppm) in the PAH-contaminated lysimeter replicates. Initial concentrations were derived from eight soil cores taken within 1 month following the supplemental naphthalene addition on day 135. Profiles of final concentrations were obtained from a single sampling consisting of nine cores taken on day 474. The top three sections represent the 92 cm treatment zone. The bottom section represents a 30 cm soil layer directly beneath the treatment zone.

trations that varied widely with position and depth, indicating that contaminants were not homogeneously distributed throughout the treatment zone (Figure 7). In lysimeter 1, naphthalene concentrations ranged from 0.04 to 192 ppm, with a mean value of $8.33 (\pm 20.07)$ ppm, lysimeter 2 produced a mean naphthalene concentration of $109.22 (\pm 206.85)$ ppm (range = 0.06–663 ppm), lysimeter 4 produced a mean naphthalene concentration of $208.09 (\pm 160.81)$ ppm (range = 0.08–600 ppm), and lysimeter 6 produced a mean naphthalene concentration of $71.68 (\pm 112.64)$ ppm (range = 0.04–391 ppm). The number of soil cores was therefore increased from 2 to 3 cores per sampling to 5–9 cores per sampling to establish a more relevant profile of soil contaminant diversity. Analysis of total petroleum hydrocarbon concentrations showed similar heterogeneous distributions (data shown in ref 2).

A precise assessment of contaminant remediation effectiveness was not derived because of the heterogeneity of contaminants within each lysimeter. However, comparisons between initial contaminant concentrations in lysimeters 1, 2, and 6 following the supplemental oil addition and final contaminant concentrations determined on day 474 indicate that naphthalene levels within the treatment zone decreased, in most cases rather substantially (Figure 7). Although bioremediation by *P. fluorescens* HK44 accounts for some of this contaminant degradation, natural attenuation by other PAH degraders and air stripping are processes that also affected PAH concentrations, as exemplified in control lysimeter 6 where naphthalene loss occurred in the absence

of HK44 microbes. Several indigenous microorganisms capable of utilizing naphthalene as a carbon source were isolated from the PAH-contaminated soils. Although not what we had originally planned or desired, the uneven distribution of pollutants provided a more representative situation of an actual contaminated site, and likely proved to be a better "real-world" test of the biosensing capabilities of *P. fluorescens* HK44.

Bioluminescent Response from *P. fluorescens* HK44.

Biosensor Detection of Volatile PAH Contaminants. The alginate-encapsulated HK44 biosensors and the multiplexed, fiber optic-based light detection system successfully detected the presence of volatile PAHs in the HK44 inoculated/PAH-contaminated soils of lysimeters 1 and 2 (Figure 8). Each of these lysimeters contained four biosensors located in the treatment zone at depths of 105, 120, 138, and 152 cm from the top of the uncontaminated soil cap. Lysimeter 3, containing no PAH contaminants, contained four similarly placed biosensors to detect background induction responses from the alginate encapsulated HK44 cells. Background bioluminescence typically remained below 800 counts/s. After the supplemental oil addition on day 135, biosensors in PAH-contaminated soils produced light signals well above background levels, approaching levels as high as 166 000 counts/s. The response profiles generally increased rapidly then gradually declined back to baseline after approximately 5 days. The decrease in signal was due to cell death (as determined by plating), likely caused by nutrient depletion and/or dehydration. Interestingly, all biosensors analyzing

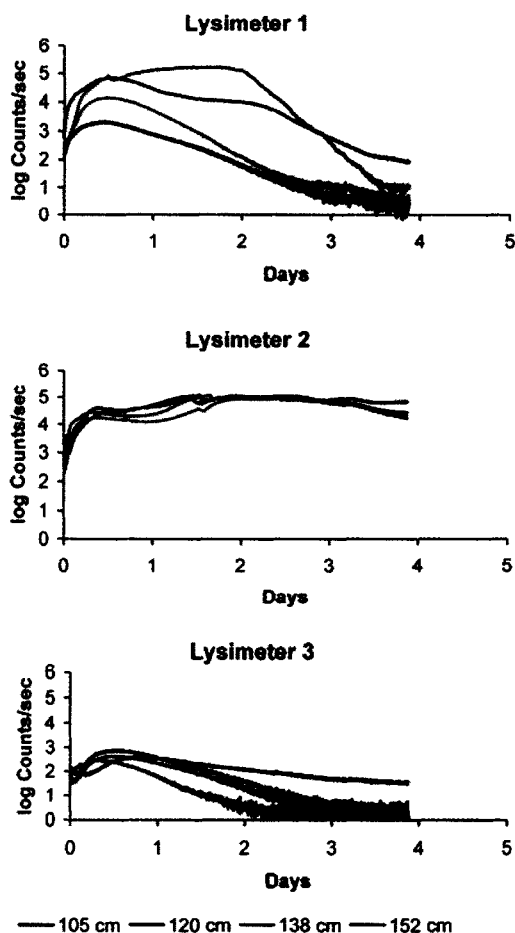


FIGURE 8. Representative biosensor response to vapor phase PAH contaminants. Results are from four biosensors suspended at varying depths in PAH-contaminated lysimeters 1 and 2 and in uncontaminated lysimeter 3. Day 0 on graphs represents day 169 of experiment. Depths originate from soil surface.

PAH-contaminated soils produced similar profiles, even though lysimeter 1 had severe distribution problems caused by channeling and pooling of contaminants (Figure 7). Thus, the volatile naphthalene compound remained distributed throughout the soil bed, probably with the aid of the air distribution system.

Direct Detection of Bioluminescence from Soil. Bioluminescence detection from soil was attempted using both buried fiber optic cables and a portable PMT device. The buried fiber optic cables were ineffective at detecting HK44 bioluminescence at population and/or activity levels found in this study. Consequently, a more sensitive portable PMT system that could be lowered into the treatment zone was designed and was ready for use on day 444. Experiments were designed to challenge the soil communities that included HK44 in the treatment zones beneath Plexiglas windows to determine if conditions were favorable for growth and biodegradation, as indicated by bioluminescence. Localized areas of soil were challenged with water and naphthalene, inorganic nutrient medium and naphthalene, or inorganic nutrient medium alone. The localized supplements were added to the soil prior to installation of pipes with and without attached Plexiglas windows.

In lysimeter 4, representing inoculated/PAH-contaminated soil, localized addition of water and naphthalene stimulated bioluminescence, typically within 24 h, with a mean intensity of $240 (\pm 100)$ counts/s over an approximate 2 day period ($n = 4$). Addition of inorganic nutrient solution alone produced similar bioluminescent signals, with a mean

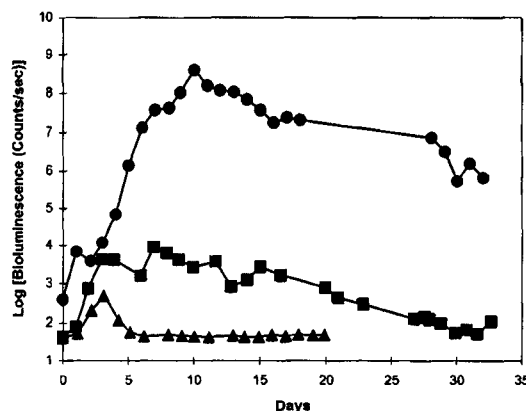


FIGURE 9. Monitoring of bioluminescence from *P. fluorescens* HK44 cells directly from soil in PAH-contaminated lysimeter 4 using the portable photomultiplier tube. Bioluminescent response occurring after addition of inorganic nutrient medium and naphthalene to soil beneath Plexiglas windows (■). Bioluminescent response in duplicate experiment except in the absence of Plexiglas windows (●). Background bioluminescence in response to the addition of inorganic nutrients alone (▲). Day 0 corresponds to day 444 of experiment.

maximum intensity of 278 counts/s and a mean duration of 2 days ($n = 2$). However, signals were not detected until 2 days after inoculation. When stimulated with the addition of both naphthalene and inorganic nutrient medium, HK44 cells produced much more intense bioluminescent signals that were initiated within 24 h and persisted in some cases for over 28 days (Figure 9). The mean maximum intensity was $4300 (\pm 1200)$ counts/s with a mean duration of $13 (\pm 5)$ days ($n = 5$). Longer bioluminescent events were observed in winter months when the soil temperatures were cooler. At the end of this period, HK44 populations in soil beneath the Plexiglas windows were determined to be $7.8 (\pm 1.8) \times 10^4$ cfu/g, an increase of 1 order of magnitude above the overall HK44 population of $8.0 (\pm 4.0) \times 10^3$ cfu/g, as determined on day 412. HK44 population estimates after the naphthalene/water additions averaged $6.8 (\pm 3.3) \times 10^3$ cfu/g, comparable to the mean population estimates. In duplicate experiments where windows were not attached to the pipe, localized HK44 populations dramatically increased 5 orders of magnitude to 7.0×10^8 cfu/g soil and maximum bioluminescence ranged from 2×10^7 to 4×10^8 counts/s, generating enough light that could be observed with the naked eye. No bioluminescence above background was observed in control lysimeter 6 under any of the localized treatment conditions.

Discussion

The release of *P. fluorescens* HK44 into soil lysimeters was initiated in order to address two basic questions pertinent to the utilization of GEMs in bioremediation processes.

(1) Can a GEM Be Successfully Introduced and Maintained in a Soil Ecosystem? The inoculation and maintenance of *P. fluorescens* HK44 within the soil ecosystem was successful. Populations rapidly declined in all inoculated soils during the first 12 days after HK44 introduction followed by a slower, more gradual decline during the remainder of the study. At no time under any of the treatments did HK44 populations approach extinction. Rather, after 444 days in the soil, HK44 cells remained in a physiological state conducive to reactivation and regrowth upon exposure to PAH contaminants and an inorganic nutrient amendment.

The initial rapid decline in HK44 numbers was not unexpected. Crozat et al. (14) observed that the inoculation of a bacterial population in soil is often followed by a decline in viable cell numbers to a characteristic survival concentra-

tion, independent of initial inoculum size. Why this distinctive reduction in cell concentration occurs cannot be attributed to a specific source but rather involves numerous abiotic and biotic factors that impinge on cellular survival rates in soil ecosystems (14).

The long-term survival of a GEM population in soil, however, does not adhere to a characteristic pattern. Van der Hoeven et al. (15), using a computer model to estimate long-term GEM survival rates after environmental release, predicted that a 3 year period was attainable. However, when reviewing actual field release studies, it is evident that a single modeling scheme cannot be successfully applied. GEMs used as agricultural inoculants have been recovered from soil from up to 4 (16) to 6 (17) years after their initial release, while in other cases, they could not be detected within 1 year after soil introduction (18, 19). In outdoor pot experiments, a *Pseudomonas putida* strain capable of methyl benzoate degradation and a biologically contained engineered derivative demonstrated relatively poor survival in bulk soil or rhizosphere environments (<100 days) regardless of the presence of the selectable substrate or the control gene (20). Again, predicting and modeling long-term GEM survival rates must take into account interactions occurring among numerous environmental factors. The overall heterogeneity of soil must also be taken into account. Whereas one area of soil may possess factors conducive to cell growth, an adjacent area may effect growth inhibition (21). In PAH-contaminated soils, additional parameters are present, such as contaminant concentrations that are too low to adequately support cell growth or too high as to exert toxic effects on the introduced population (21, 22). Successful colonization by a GEM will therefore always rely on a complex balance between the above factors, with controllable factors, such as soil aeration or moisture content, being carefully planned into the overall design of the field release site.

(2) Are Bioreporter Organisms Capable of Detecting and Monitoring the Overall Bioavailability and Biodegradation of an Environmental Contaminant? *P. fluorescens* HK44 successfully provided real-time data that reflected naphthalene bioavailability, degradative activity, and optimal degradation conditions. Biosensors, utilizing alginate encapsulated HK44 cells, monitored for the presence of naphthalene in the vapor phase, while a portable PMT monitored for actively bioluminescing HK44 cells directly from soil. In the biosensors, environmental factors such as temperature and moisture content affected cell viability within the encapsulation matrix, and, therefore, a quantitative assessment of vapor phase naphthalene concentrations could not be accurately determined. Typical survival rates of HK44 cells in the biosensors extended to approximately 1 week, whereupon they would be replaced with newly encapsulated cells. For periodic monitoring of a contaminated site or postremediation monitoring, such biosensors are capable of providing a rapid assessment of contaminant bioavailability. However, new encapsulation materials for increasing cell longevity are currently being studied in our laboratory in order to develop biosensors capable of long-term, online monitoring of vapor and aqueous phase contaminants. Additionally, new strains of bioluminescent bioreporters are continually being engineered with improved sensitivity, increased levels of bioluminescence, and greater genetic stability (23–29).

The PMT detection system was capable of detecting bioluminescence directly from soil-borne HK44 cells. Initial use of the PMT occurred after day 400, when naphthalene concentrations and HK44 populations were too low to generate measurable bioluminescent responses. It was therefore necessary to supplement localized test areas with naphthalene and inorganic nutrients. Results demonstrated that HK44 cells could be revived under these conditions to

produce bioluminescence at magnitudes well above PMT detection limits, persisting for up to 28 days. Addition of inorganic nutrients or naphthalene alone did not induce bioluminescence, indicating that the soil not only contained little or no naphthalene but also was limited by an ingredient(s) in the inorganic nutrient medium. Most importantly, however, studies performed in the absence of Plexiglas windows demonstrated that oxygen was a limiting factor, and, if available, could substantially restimulate HK44 populations to approximately 1×10^9 cfu/g soil.

P. fluorescens HK44 represents the first recombinant microorganism to undergo full U.S. EPA biotechnology risk assessment review and achieve environmental release status for applications in bioremediation. Although the environmental research testing of this microbe was conducted under replicated and controlled conditions, it is expected that the test scale would adequately simulate open environmental release to subsurface vadose if the organism could be delivered to that environment. Furthermore, upon environmental introduction, results from this investigation clearly demonstrate that recombinant microorganisms with application for in situ bioremediation can be viably maintained. While decay in population densities does occur, environmental manipulation can result in regrowth. In the case of *P. fluorescens* HK44, this growth and activity is quantifiable through online bioluminescent monitoring. It is perhaps not surprising that the primary carbon source and electron donor (PAHs) as well as electron acceptor (oxygen) appear fundamentally limiting in terms of in situ metabolic activity, growth, and aerobic biodegradative processes of the organism. Thus, even as a surrogate for indigenous PAH degrading microbes, HK44 or other bioaugmentative strain addition poses two challenges for the bioremediation practitioner: (1) adequate dissemination of the microorganism in the subsurface environment and (2) overcoming mass transfer limitations of electron donors and acceptors controlling the degradative response. In either instance, the use of bioluminescent sensor organisms such as HK44 may provide a new analytical tool for monitoring and controlling biodegradative processes in response to critical operating conditions.

Acknowledgments

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Bioluminescent bioreporter integrated circuits: potentially small, rugged and inexpensive whole-cell biosensors for remote environmental monitoring

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1. SUMMARY

A bioluminescent bioreporter integrated circuit (BBIC) is a novel whole-cell biosensor that combines the environmental monitoring capabilities of genetically engineered bioluminescent micro-organisms (bioreporters) with optical application-specific integrated circuits. A BBIC device consists of bioreporters sustained within a micro-environment, the integrated circuit microluminometer, and a light-tight enclosure. The bioreporter typically contains the *luxCDABE* reporter genes encoding the enzymes required for bioluminescence. In the presence of a targeted analyte, a gene (transcriptional) regulatory system induces the expression of the *luxCDABE* genes. Analytical benchmark data for exposure of the bioreporter *Pseudomonas fluorescens* 5RL to salicylate was determined using a flow-through test system. The detection limit (after a 45 min exposure) was *ca* 50 $\mu\text{g l}^{-1}$ and response times decreased from *ca* 45 to 20 min as the concentration increased from 50 $\mu\text{g l}^{-1}$ to 1 mg l^{-1} . These results are currently being used to scrutinize enclosures and micro-environment configurations to develop a field deployable BBIC. A successful BBIC could provide a simple and inexpensive means of creating a 'laboratory-on-a-chip' and could be used in a network to protect valuable

human and environmental resources. This article reviews the present state of *luxCDABE*-based bioreporter research, demonstrates the integration of the bioreporters with complementary metal oxide semiconductor photodiode-integrated circuits, and discusses future challenges for real-time *in situ* BBIC environmental monitoring.

2. INTRODUCTION

Safeguarding human and environmental resources against harmful agents requires the development of new *in situ*, real-time monitoring devices that can be easily deployed at multiple strategic locations. Ideally, a device would be able to detect a pollutant, toxic chemical, or warfare agent rapidly, at relevant concentrations, and in a cost-effective manner. Many conventional analytical technologies (e.g. spectrometry) with enough selectivity to identify trace amounts of specific analytes are too expensive to be deployed in a multi-sensor network and/or cannot be used in real-time monitoring because they require sample preparation steps prior to detection. Biosensor technology is an analytical option that can circumvent these problems by providing rapid, inexpensive, sensitive, and in some cases selective analysis of environmental samples (Collings and Caruso 1997; Matrubutham and Saylor 1998; Nistor and Emneus 1999; D'Souza 2001; Keane *et al.* 2002; Marazuela and Moreno-Bondi 2002).

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A biosensor is an analytical device that uses biological macromolecule(s) to recognize an analyte and subsequently activate a signal that is detected with a transducer. The transducer typically converts the biological response into an electrical signal. The biological recognition of the analyte affords biosensors excellent sensitivity (typically $\mu\text{g l}^{-1}$ to mg l^{-1}) and selectivity for the detection of an individual compound, a group of similar molecules (typically based on similar functionality and molecular shape) or a general toxic effect. Biological recognition elements are typically enzymes, antibodies, DNA, receptors, or regulator proteins and can be used either in whole-cells or isolated and used independently (D'Souza 2001). Biosensors using purified biomolecules can have rapid response times, but purification procedures can be time-consuming and expensive (Daunert *et al.* 2000). Permeabilized nonviable cells have been used as an inexpensive alternative to provide biological recognition elements (D'Souza 2001). However, both purified biological recognition elements and permeabilized whole-cells mostly have been applied in laboratory settings or in point-of-use determinations and are probably less useful for long-term monitoring of environmental systems because of the degradation of the recognition element with time.

Exploiting living micro-organisms in whole-cell biosensors (sometimes referred to as microbial biosensors) is beneficial as micro-organisms are continually synthesizing complex systems of biomolecules (including biological recognition elements) required for sensing and reacting to environmental changes. Micro-organisms are also ubiquitous and it is highly probable that a strain can be acquired to survive within the operating parameters of a given environment. Micro-organisms contain or can be genetically engineered to contain reporter gene(s) that expresses reporter protein(s) responsible for detectable signals. Furthermore, these reporter micro-organisms (referred to herein as bioreporters) can support multi-enzyme reactions, and allow more complicated biological recognition and signalling schemes for biosensors. Cofactors and cosubstrates for enzyme reactions can also be provided by bioreporters, thereby eliminating the need for adding exogenous reagents to produce signals. Bioreporters also have the ability to determine the bioavailability of an analyte instead of the total concentration, which can include chemical species of the analyte that are not physiologically relevant (e.g. insoluble) (Heitzer *et al.* 1992; Alexander 2000; Rasmussen *et al.* 2000). Limitations of the use of bioreporters include the facts that living bioreporters require nutrients to survive and can require minutes to produce a detectable signal because of the time needed for mass transfer processes and in some cases, the time required for the expression of the protein(s) responsible for the biological signal.

A bioluminescent bioreporter integrated circuit (BBIC) is a unique whole-cell biosensor that uses an integrated circuit

optical transducer to detect blue-green light (*ca* 490 nm) emitted by a genetically engineered bioreporter in response to a specific stimulus (Simpson *et al.* 1998; Simpson *et al.* 2001; Bolton *et al.* 2002). Conceptually, the BBIC device consists of a light-tight enclosure, a bioluminescent bioreporter strain, a micro-environment that sustains the living bioreporter cells, and the integrated circuit for light detection and signal processing (Fig. 1). During the detection process, a targeted analyte diffuses to the bioreporters within the micro-environment and interacts with the gene regulator protein of the bioreporter. This concentration-dependent interaction induces the expression of the *luxCDABE* genes that code for the proteins that produce light. The bioluminescence is then detected with an integrated circuit microluminometer and the signal relayed to a central database via wireless communication for potential mitigation.

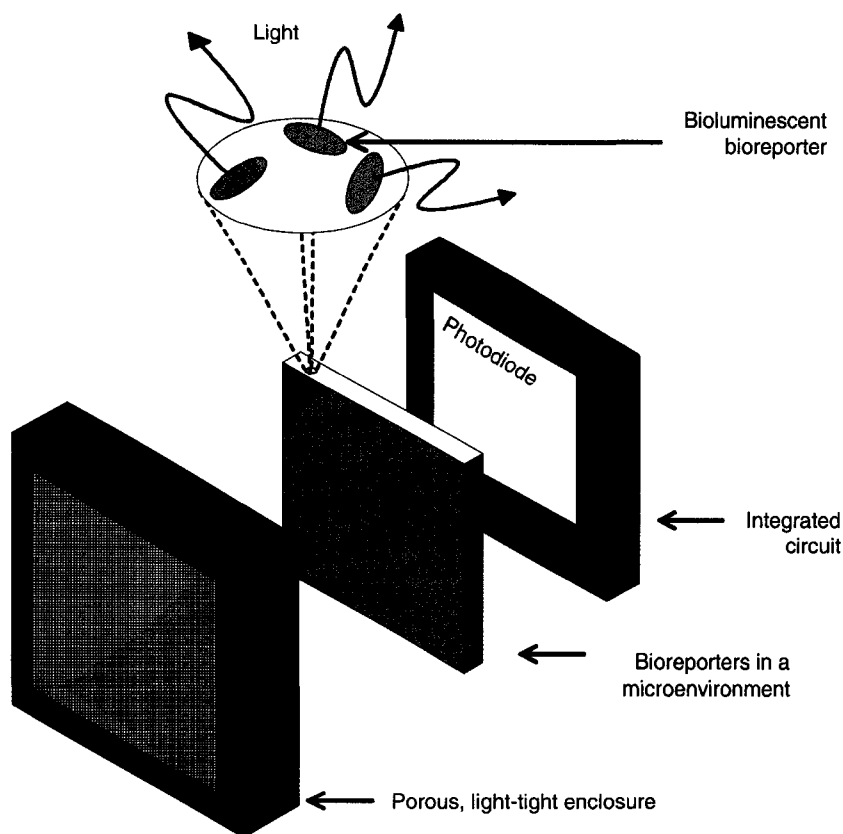
This article discusses and reviews the development of the bioluminescent bioreporter and light-detecting integrated circuit technologies, and the problems and current limitations associated with interfacing the bioreporter with microluminometers. The ultimate goal of the BBIC technology is the creation of a family of 'laboratory on a chip' biosensors for remote-sensing environmental applications.

3. BIOLUMINESCENT BIOREPORTER TECHNOLOGY

3.1 Bioreporters

A bioreporter (sometimes referred to as a reporter cell) is a living organism that produces a measurable signal when it senses a particular chemical or a physical change in its environment. A bioreporter typically contains a gene (transcriptional)-regulatory system coupled to a reporter gene(s) that encodes for a reporter protein(s) responsible for the signal. These reporter and/or regulator genes can be native to the cell or acquired by genetic transformation. A common type of transcriptional regulatory system consists of a regulator gene(s), its expressed regulator protein(s) and the promoter/operator DNA sequence that controls the expression of downstream gene(s) (Fig. 2). These elements work in concert to sense environmental changes and control the expression of a gene or an operon to enhance the physiology of the cell. For example, the LysR-type transcriptional regulatory system uses positive control of induction to activate gene expression (Fig. 2). With this system, an analyte acting as the inducer forms a complex with the regulator protein. This complex interacts with the promoter to initiate the binding of RNA polymerase and the transcription of downstream genes (Fig. 2). By fusing a native promoter to the reporter gene(s) and inserting the fusion into a host cell, the resultant bioreporter is engineered to harness the native transcriptional regulatory system for the purpose of signalling

Fig. 1 Conceptual diagram of the key components that make up a bioluminescent bioreporter integrated circuit (BBIC). The light-tight enclosure is used to block environmental light, provide diffusion-limited chemical contact with the outside environment, house the micro-environment and seal the electronics. The micro-environment supplies nutrients for the bioreporters, provides protection for the cells and in some cases may contain an encapsulation matrix. The integrated circuit detects the bioluminescence emitted by the bioreporters, processes the signal, and communicates the results to a central database. An application specific BBIC will also contain a battery that is not shown



that the native protein is being expressed and/or that the targeted environmental change has occurred.

Several reporter genes have been isolated from a variety of naturally occurring organisms (Daunert *et al.* 2000) and have been categorized by the means of detection (Kohler *et al.* 2000; Keane *et al.* 2002). Reporter genes coding for proteins that produce colour changes, fluorescent molecules, electro-active species, or bioluminescence have been discovered and can be detected by conventional analytical devices.

Colorimetric, fluorescent and electrochemical monitoring can require significant amounts of power, a limited resource in remotely deployed sensors. For example, colorimetric and fluorescent devices require power-draining sources of radiation (e.g. lasers, lamps or light emitting diodes) to excite the analyte for detection. Furthermore, environmental samples contain many compounds that can interfere with electrochemical, colorimetric and fluorescent detection. Some colorimetric and fluorescent signalling products also can remain active for long periods of time (even persisting after cell death), and thus are not conducive to dynamic real-time environmental monitoring. For example, the native green fluorescence protein (gfp) persists until cell lysis (Tombolini *et al.* 1997); however, variants have been made that have half-lives of 40 min to a few hours (Andersen *et al.* 1998).

In contrast, detection of bioluminescence requires no external source of radiation, as light is produced by biochemical reactions. This simple analytical strategy also minimizes the background noise and requires no wavelength discrimination. As bioluminescence requires energy in the form of high-energy biomolecules, analytes that induce the *lux* system and provide energy can be used for dynamic monitoring applications. In a study that compared the responses of fluorescent (gfp and dsred proteins) and bioluminescent (*luxCDABE* and *luc FF* genes) *Escherichia coli* plasmid-based bioreporters, bioluminescence had faster response times and lower detection limits than the fluorescence signals (Hakkila *et al.* 2002).

3.2 Bioluminescence

A variety of both eukaryotic and prokaryotic species have genes that code for proteins that synthesize bioluminescence-generating products in the presence of O₂ and high-energy biomolecules. Certain eukaryotic species including fungi, insects, ctenophores, coelenterates and dinoflagellates contain *luc* or *ruc* genes that express a luciferase enzyme that catalytically oxidizes a substrate(s) (luciferin) to generate light (Wilson and Hastings 1998). Natively, the oxidized luciferin is recycled for further use by the oxidation of

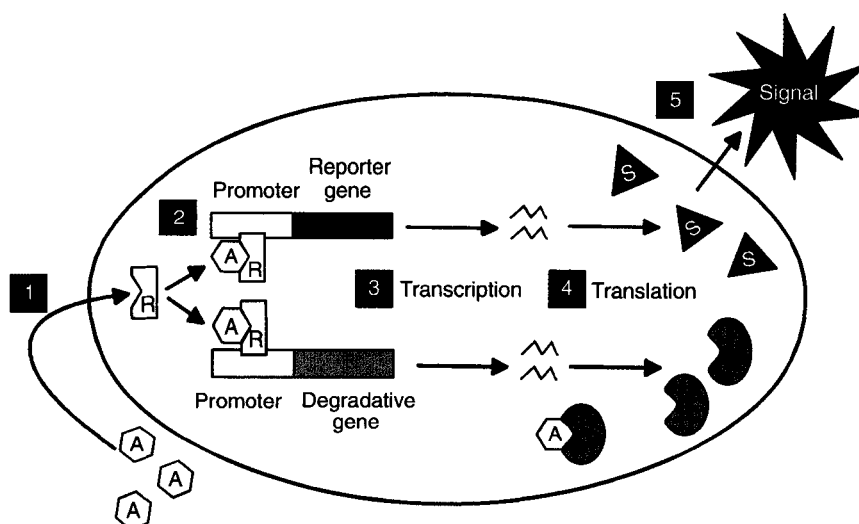
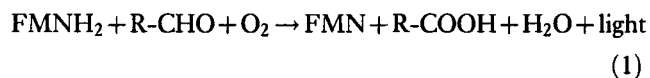


Fig. 2 Conceptual diagram of a bioreporter with a LysR-type transcriptional regulatory system showing the steps involved in gene expression and signalling. The bioreporter cell contains a positively controlled transcriptional regulator system that induces both the reporter gene, and in this case, the genes encoding the protein that catabolize the analyte. A is the analyte, R designates the regulator protein, D is the degradative enzyme, and S is the signalling protein. The regulator gene is constitutively expressed and not shown. The detection process begins when the analyte, A, enters into the cytoplasm of the cell and forms a complex with the regulator protein, R (step 1). The complexes interact with the promoters to induce the expression of the genes (step 2). The genes are transcribed to produce mRNAs (step 3) and the mRNAs are translated to produce signalling proteins and enzymes to degrade the analyte (step 4). The signalling proteins can be directly detected or catalyze reactions to produce the detected signal (step 5). *Pseudomonas fluorescens* HK44 responds to salicylate in this manner. The bioreporter *Ps. fluorescens* 5RL contains a translational insertion of the reporter genes into the degradation gene, rendering the degradative pathway inactive and allowing the analyte to accumulate within the cell. This type of bioreporter is useful for an early-warning BBIC

common high-energy biomolecules such as ATP. Prokaryotes including species in the genera *Vibrio*, *Photobacterium* and *Photorhabdus* activate a *luxCDABE* gene cluster to produce the enzymes required for bioluminescence (Meighen 1991; Meighen 1994). The *luxA* and *luxB* genes code for a heterodimeric luciferase (mono-oxygenase), the *luxC* gene for a reductase, the *luxD* for a transferase and the *luxE* for a synthetase. The reductase, transferase and synthetase form a complex to convert a fatty acid in an acyl carrier protein to an aldehyde at the expense of an ATP and an NADPH molecule. In the presence of oxygen, both the aldehyde and an FMN₂ molecule are cosubstrates that are oxidized by the luciferase to generate light (eqn 1).



These substrates are typically not referred to as luciferins, as they are common cell metabolites or are derived from ordinary metabolites after minimal modification. The *luxG* gene is also found in luminescent species of bacteria and may play a role as a flavin reductase in the recycling of the FMN₂ (Zenno and Saigo 1994). The three most common variants of the *luxAB* gene isolated from *Vibrio fischeri*, *V. harveyi*, and *Photorhabdus luminescens* appear to have usable

thermal stability to 30, 37 and 42°C, respectively (Meighen 1991), with *P. luminescens* having a half-life of 3 h at 45°C (Meighen 1991). Furthermore, the entire *luxCDABE* operons for *V. harveyi* and *P. luminescens* have strong signals when expressed in *E. coli* at 37°C (Szittner and Meighen 1990).

The *lux*, *ruc* and *lux* genes have been used to create bioluminescent bioreporter strains (Daunert *et al.* 2000). The use of the eukaryotic reporter genes in foreign hosts typically requires the exogenous addition of the luciferin to generate light. A similar approach is sometimes used in bacterial systems using the *lux* system; the *luxAB* genes are expressed and an aldehyde is added exogenously. Use of the *luxCDABE* (the *luxG* is not included in most constructs) gene cluster offers advantages because the expressed proteins produce bioluminescence without the addition of exogenous substrates. Bioreporters using the *luxCDABE* cluster typically can be (i) naturally occurring environmental isolates, (ii) genetic constructs with constitutive promoters (always expressed), (iii) constructs with promoter-*lux* fusions for specific stress-related responses, (iv) constructs with gene regulatory systems that are specific for certain analytes or classes of analytes and (v) multiple constructs with different promoters functioning in a genomic-wide array.

3.3 *luxCDABE* Bioreporters

Naturally occurring bioluminescent bioreporter strains are used to assess the toxicity of a sample to cells (Bulich 1982; Kaiser and Palabrica 1991). Cultures of bioluminescent bacteria are mixed with various concentrations of a sample containing a potential toxic reagent and the bioluminescence is determined. In the presence of a toxin, the bioluminescence is attenuated either by loss of metabolic activity or cell death. By comparing the bioluminescence with a control sample that was not exposed to the toxin, the magnitude of the decrease is calculated and used to quantify toxicity. A number of different commercially available systems exist that provide the necessary reagents, freeze- or liquid-dried bioreporters and instrumentation to observe bioluminescence (Jennings *et al.* 2001). These systems are used to determine the toxicity of pure compounds and complex samples like wastewater effluents. Although a signal decrease is attributed to toxicity and provides evidence that a sample contains a toxic agent, the true source of a diminished signal is difficult to ascertain, as a number of mechanisms other than toxicity (including lack of energy or oxygen) can attenuate bioluminescence (Cronin and Schultz 1998).

Bacterial strains other than naturally occurring hosts have been genetically engineered to act as bioluminescent bioreporters for the sensing of toxicity caused by pollutants or environmental stressors. For example, Kelly *et al.* (1999) constructed a bioluminescent bioreporter by insertion of the constitutively expressed *luxCDABE* genes (*V. fischeri*) into a *Pseudomonas fluorescens* strain isolated from a wastewater treatment facility. The bioluminescent bioreporter (designated Shk1) was used to estimate the toxicity of wastewater, the environment from which it was isolated. This overcomes a drawback of commercial assays using the marine micro-organism *V. fischeri*, which requires the addition of high salt medium to freshwater samples prior to testing (Lajoie *et al.* 2002). It was suggested that salt can alter the chemistry and thus the toxicity of a freshwater sample.

Promoters for stress response genes have been fused to the *luxCDABE* cassette to produce bioreporters that respond to a number of different types of environmental and chemical stresses (Table 1). These types of bioreporters are not used to identify a particular stressor, but simply that the stress response has been activated. For example, heat shock promoters in bioreporters have been used to respond not just to conditions involving a change in temperature, but also to detect exposure to chemical and biochemical stressors such as antibiotics, organic compounds, oxidative reagents and heavy metals (Kregel 2002). For example, Belkin *et al.* (1997) constructed five plasmids containing different promoter-*luxCDABE* fusions and used the plasmids to transform an *E. coli* strain into a panel of bioluminescent

bioreporters for monitoring stress responses. Promoters for *katG* and *soxRS* for oxidative stresses, *recA* for DNA damage, *grpE* for the heat shock/protein damage system and *fabA* involved in fatty-acid synthesis inhibition were used. The bioreporters reacted specifically to stressors within their respective groups, except for phenol, which nonspecifically induced all of the strains. Davidov *et al.* (2000) offered potential improvements in the SOS-type *recA* promoter-*luxCDABE* bioreporter response by constructing strains that (i) deactivated the expression of an efflux pump (*tolC* mutation) to improve sensitivity, (ii) provided a more stable construct by incorporating the promoter-*lux* fusion into the chromosome and (iii) used the *luxCDABE* genes from *P. luminescens*, allowing use at higher temperatures. A plasmid containing an SOS dependent promoter-*luxCDABE* fusion (the *lux* genes were isolated from *Photobacterium leiognathi*) has been introduced into the *Salmonella typhimurium* TA1535 that was used in the Ames test for mutagenesis (Rabbow *et al.* 2002). The system gave comparable results to the MutatoxTM (Strategic Diagnostics Inc., Newark, NJ, USA), Ames and SOS-chemotests assays.

Bioreporters containing a promoter-*luxCDABE* gene fusion have been used selectively to monitor for the presence of inorganic and organic analytes (Table 1). Genetic strategies to engineer this type of bioreporter involve (i) isolating micro-organisms that can express proteins that selectively degrade, bind, sequester, transport or transform the targeted analyte, (ii) determining the promoter sequence that regulates the selective function, (iii) fusing the promoter sequence to the *luxCDABE* cassette and (iv) inserting the promoter-*luxCDABE* fusion into a host strain containing the gene regulatory system. For example, Corbisier *et al.* (1999) developed a plasmid-based *Alcaligenes eutrophus* bioreporter containing the promoter for the *pbr* operon (efflux pump) fused to the *luxCDABE* genes from *V. fischeri*. The bioreporter was able to selectively detect lead, but not other potential interfering metals such as cadmium, copper, zinc and mercury. Analogous bioreporters using the *luxCDABE* genes have been developed to sense cadmium, chromate, cobalt, copper, iron, mercury, nickel and zinc (Table 1) (Rensing *et al.* 1999; Ramanathan *et al.* 1997).

Bioreporters also have been developed to sense specific organic pollutants using promoter-*luxCDABE* fusions. Generally, the promoter that activates the genes coding for catabolism of the analyte is used to construct the bioreporter. A pioneering example of this type of bioreporter is *Ps. fluorescens* HK44 that was developed to monitor the biodegradation of naphthalene (King *et al.* 1990). Genes that code for the enzymes catabolizing naphthalene are contained on a large plasmid, pUTK21, in two separate operons coding for the upper (*nah*) and lower (*sal*) pathways, respectively. The upper pathway converts naphthalene to

Table 1 *luxCDABE* bioreporters

Analyte (effector)	Promoter	Micro-organism	Reference
Nonspecific stress response			
DNA damage (mitomycin)	<i>recA, uvrA, alkA</i>	<i>E. coli</i>	Vollmer <i>et al.</i> (1997)
Gamma-irradiation	<i>recA</i>	<i>E. coli</i> DPD2794	Min <i>et al.</i> (2000)
Genotoxins	<i>cda</i>	<i>E. coli</i>	Ptitsyn <i>et al.</i> (1997)
Heat shock	<i>dnaK, grpE, lon</i>	<i>E. coli</i>	Van Dyk <i>et al.</i> (1995); Rupani <i>et al.</i> (1996)
Organic toxins			
	<i>recA</i>	<i>E. coli</i> and <i>S. typhimurium</i>	Davidov <i>et al.</i> (2000)
Oxidative stress	<i>katG</i>	<i>E. coli</i>	Belkin <i>et al.</i> (1996)
Various toxicants	<i>katG, micF, recA, grpE, fabA</i>	<i>E. coli</i> DPD2794	Belkin <i>et al.</i> (1997)
Various toxicants	<i>katG, micF, fabA, lon, uspa</i>	<i>E. coli</i>	Ben-Israel <i>et al.</i> (1998)
Ultrasound	<i>fbaA, grpE, katG, recA</i>	<i>E. coli</i>	Vollmer <i>et al.</i> (1998)
Ultraviolet light	<i>recA</i>	<i>Ps. aeruginosa</i>	Elasri and Miller (1998)
Metals			
Cadmium	Not specified	<i>R. eutrophus</i> AE1239	Corbisier <i>et al.</i> (1996)
Chromate	<i>chr</i>	<i>R. eutrophus</i> CH34	Peitzsch <i>et al.</i> (1998)
Cobalt	<i>chr</i>	<i>R. eutrophus</i> CH34	Tibazarwa <i>et al.</i> (2000)
Copper	<i>cup 1</i>	<i>E. coli</i>	Holmes <i>et al.</i> (1994); Daunert <i>et al.</i> (2000)
Heavy metals			
Iron	<i>zntA, copA</i>	<i>E. coli</i>	Riether <i>et al.</i> (2001)
Lead	<i>pupA</i>	<i>Ps. putida</i>	Khang <i>et al.</i> (1997)
Mercury	Not specified	<i>R. eutrophus</i> AE1239	Corbisier <i>et al.</i> (1996)
Nickel	<i>mer</i>	<i>E. coli</i>	Selifonova <i>et al.</i> (1993)
Zinc	<i>cnr</i>	<i>R. eutrophus</i> CH34	Tibazarwa <i>et al.</i> (2000)
	<i>smtA</i>	<i>Synechococcus</i> PCC7942	Erbe <i>et al.</i> (1996)
Organic compounds			
2,4-Dichlorophenol	<i>tdfDII</i>	<i>R. eutropha</i> JMP134	Hay <i>et al.</i> (2000)
3-Xylene	<i>xyl</i>	<i>Ps. putida</i>	Burlage (1998)
4-Chlorobenzoate	<i>fcbA</i>	<i>E. coli</i>	Rozen <i>et al.</i> (1999)
BTEX (benzene, toluene, ethylbenzene, xylene)	<i>tod</i>	<i>Ps. putida</i> TVA8	Applegate <i>et al.</i> (1998)
Isopropyl benzene	<i>ipb</i>	<i>Ps. putida</i> RE204	Selifonova and Eaton (1996)
Naphthalene	<i>nahG</i>	<i>Ps. fluorescens</i> HK44	King <i>et al.</i> (1990)
Organic peroxides	<i>katG</i>	<i>E. coli</i>	Belkin <i>et al.</i> (1996)
PCBs	<i>orfO-bphA</i>	<i>R. eutropha</i> ENV307	Layton <i>et al.</i> (1998)
<i>p</i> -chlorobenzoic acid	<i>fcbA</i>	<i>E. coli</i>	Rozen <i>et al.</i> (1999)
<i>p</i> -cymene	<i>cymB</i>	<i>Ps. putida</i>	Ripp <i>et al.</i> (in press)
Salicylate	<i>nahG</i>	<i>Ps. fluorescens</i> HK44	King <i>et al.</i> (1990)
Trichloroethylene	<i>tod</i>	<i>Ps. putida</i> TVA8	Shingleton <i>et al.</i> (1998)
Biological importance			
Genome	Various	<i>E. coli</i>	Van Dyk <i>et al.</i> (2001a); Van Dyk <i>et al.</i> (2001b)
Ammonia	<i>hao</i>	<i>N. europaea</i> Km ^r <i>hao-lux</i>	Simpson <i>et al.</i> (2000)
Alginate production	<i>algD</i>	<i>Ps. aeruginosa</i>	Wallace <i>et al.</i> (1994)
Antibiotic testing against <i>Staphylococcus aureus</i> in mice	Not specified	<i>S. aureus</i>	Francis <i>et al.</i> (2000)
Haemolysin production	<i>voh</i>	<i>E. coli</i>	Bang <i>et al.</i> (1999)
<i>In vivo</i> monitoring of <i>S. Typhimurium</i> in mice		<i>S. typhimurium</i>	Contag <i>et al.</i> (1995)
<i>N</i> -acyl homoserine lactones	<i>lasRI', luxRI', rhIRI'</i>	<i>E. coli</i>	Winson <i>et al.</i> (1998)
Nitrate	<i>narG</i>	<i>E. coli</i>	Prest <i>et al.</i> (1997)
Tetracycline	<i>tetR</i>	<i>E. coli</i> pUT- <i>tetlux</i>	Hansen and Sorensen (2000)

salicylate and the lower pathway oxidizes salicylate. The *nahR* gene, located between the two operons, constitutively expresses the NahR protein (in the LysR family of

transcriptional regulators) that diffuses into the cytoplasm and positively regulates both operons. Salicylate, the degradation product of naphthalene, forms a complex with

the NahR protein and binds to the promoter, resulting in the induced expression of both sets of biodegradation genes (Schell 1985). The bioreporter *Ps. fluorescens* HK44 contains the *luxCDABE* genes from *V. fischeri* that are also under the control of the *nahRG_p* promoter and are expressed in the presence of salicylate (e.g. Fig. 2). Bioluminescence of *Ps. fluorescens* HK44 in heavily contaminated soil slurries from a manufactured gas plant site was detected using light guides coupled to a photomultiplier tube (PMT) system (King *et al.* 1990). The strain was also used in field lysimeters to study the environmental monitoring capabilities of bioreporters and the environmental fate of genetically modified micro-organisms (Ripp *et al.* 2000). Bioreporters for other organic molecules have been developed and are listed in Table 1.

In a large-scale and innovative effort, Van Dyk *et al.* (1998, 2001a,b) constructed 689 bioreporters with different promoter-*luxCDABE* gene fusions contained on plasmids creating a living array to study the genome-wide transcriptional responses of *E. coli*. Specifically, *E. coli* (DPD1675) was transformed with the plasmids containing different random *E. coli* DNA fragments that were ligated upstream of the promoterless *luxCDABE* genes. Successful bioreporter strains were selected from the transformants using sequencing data to ascertain the identity of unique promoters with the proper orientation. The array of bioreporters contained promoters for several global stress response regulons and covered *ca* 27% of the predicted transcriptional units of *E. coli* (Van Dyk 2002). The genome-wide promoter-*luxCDABE* gene fusions can be arrayed (e.g. microtiter plates) to demonstrate the overall transcriptional response of *E. coli* to physical and chemical perturbations and provided information similar to DNA arrays without the use of problematic nucleic acid isolation procedures (Van Dyk *et al.* 2001b).

4. DETECTING BIOLUMINESCENCE FROM BIOREPORTERS

Bioluminescence of bioreporters has been detected with photodiodes, avalanche photodiodes and photomultiplier tubes and imaged with photographic film silicon intensified target cameras and charge-coupled device cameras, (Simpson *et al.* 1998; Contag *et al.* 2000; Greer and Szalay 2002). As with any other optical signal, the bioluminescence can be transferred to the detectors with lenses, fibre optics (Ripp *et al.* 2000; Marazuela and Moreno-Bondi 2002), liquid light guides or monitored directly by a detector. For example, a photon counting photomultiplier probe was inserted into the subsurface of lysimeters through 4" diameter bore holes to directly monitor the bioluminescence of *Ps. fluorescens* HK44 in polyaromatic hydrocarbon-contaminated soils (Ripp *et al.* 2000; Sayler *et al.* 2001).

This detection system was portable, though it required an external power supply and an RS232 cable for data communication.

The ideal detector for *in situ* real-time monitoring of remote environmental locations would be small and rugged, have good sensitivity, be inexpensive, and have both a global positioning system and wireless communication circuitry. Furthermore, the system should consume minimal power, as the size and mass of the battery has a great effect on the size and mass of the overall biosensor package.

Complementary metal oxide semiconductor (CMOS) technology has the ability to make electronic devices that are small, inexpensive and have low power consumption. Using CMOS, a single integrated circuit can be manufactured to contain tens of millions of transistors. Thus, a single chip can integrate many different standard electronic functions. For example, integrated circuits containing photodiodes could be equipped with signal processing, radio frequency (RF) wireless telemetry and global positioning circuits (Simpson *et al.* 1998). Ancillary functions such as temperature sensing could also be incorporated onto the sensor. Thus, integrated circuits realized with CMOS are excellent platforms for the detection of bioluminescence from bioreporters.

4.1 Developing integrated circuits for bioluminescent bioreporter detection

Initially, different types of photodiodes (p-diffusion/n-well and n-well/substrate photodiodes) and signal processing circuits were analysed with test chips to determine the optimal configuration for sensing low levels of bioluminescence (Simpson *et al.* 1998; Simpson *et al.* 2001). The n-well/substrate photodiode was determined to have greater quantum efficiency (66% at 490 nm) than the p-diffusion/n-well type and was selected for further development.

Processing of the resultant photodiode signal was accomplished using a current-to-frequency converter circuit. Briefly, electrons from the photodiode are collected with a small on-chip capacitor. A gated integrator at the input allows the collection of the photocurrent (and dark current) until the output voltage reaches a threshold value. At the threshold voltage, a pulse is generated that resets the input gated integrator by closing a switch across the capacitor. The switch is then opened and the process continues to produce pulses that may be individually timed to obtain pulse interval measurements that are inversely proportional to current. Alternatively, the pulses can be counted for a fixed period of time to provide frequency data that is proportional to the current. Using the information gleaned from the test chips, a large area (1.47 mm²) photodiode integrated circuit was produced using the 0.5 µm bulk CMOS process. The chip demonstrated large dynamic ranges and was able to

monitor bioluminescence produced by bioreporters (Simpson *et al.* 2001). Unlike PMT-based systems, the BBIC integrated circuit microluminometer can be exposed to room light without damage and can be exposed to alternating high and low light levels without a significant memory effect.

A second version of the large area BBIC chip was produced to reduce the leakage current (Bolton *et al.* 2002). Using the n-well/substrate type photodiode and the optimized current-to-frequency converter, this version of the BBIC chip demonstrated a minimum detectable signal of 0.15 fA with a 25 min integration time at room temperature (Bolton *et al.* 2002). This minimum detectable signal corresponds to the bioluminescence emitted from *ca* 5000 fully induced *Ps. fluorescens* 5RL cells (see Section 5.1).

5. INTERFACING BIOLUMINESCENT BIOREPORTERS WITH INTEGRATED CIRCUIT DETECTION

5.1 Initial testing of a bioluminescent bioreporter with integrated circuit detection

Initially, silicon chips were wire bonded to standard 40-pin ceramic chip carriers for testing. To protect the wire bonds, the chips were encapsulated in silicone elastomer, polydimethyl siloxane (PDMS – Sylgard 184; Dow Corning, Midland, MI, USA). Enclosures have been and are being designed to (i) mount to the chip carriers, (ii) block room light, (iii) allow diffusion-limited contact with the outside environment, (iv) protect the electronics and/or (v) provide containment of the bioreporters. The ceramic carriers and enclosures were mounted onto sockets attached to a printed circuit test board. A power supply was connected to the test board to provide 3.3 V power and off-chip adjustable bias voltages. The output from the integrated circuit was transformed to a pulse period, converted to a value proportional to bioluminescence recorded and displayed with time using a personal computer, a digital interface board and software.

Pseudomonas fluorescens 5RL was selected for initial analytical testing. This construct contains a salicylate-inducible regulatory system and the *luxCDABE* operon as a transpositional mutation of the *sal* operon (King *et al.* 1990). The *lux* genes were inserted into the operon that coded for the degradation of salicylate, thus preventing the expression of the degradation genes and allowing the intracellular accumulation of salicylate. The accumulation of the inducer molecule should provide a more rapid and sustained response and this genetic strategy may prove to be useful for the development of early warning type biosensors. Salicylate, an important plant biomolecule, is also a useful test analyte because it is water-soluble, nonvolatile, nontoxic and is not degraded by *Ps. fluorescens* 5RL.

Initially, flow-through enclosures were developed to monitor real-time bioluminescence from cells in aerated liquid cultures. Cells in known physiological states were pumped through the flow-cell for on-line determinations of bioluminescence, while at the same time samples were removed for off-line analysis. These studies were designed to produce analytical benchmark data for testing other types of enclosures. The flow-through enclosures house glass tubes with a 9-mm outside diameter.

Cultures were prepared by inoculating *Ps. fluorescens* 5RL cells into 1 l of sterile minimal salts medium supplemented with trace elements containing glucose as a carbon source, and rotating constantly at 170 rev min⁻¹. All experiments were performed at 20.5 ± 0.5°C as temperature affects the dark signals and the growth of the bioreporters. When the cultures reached an O.D.₅₄₆ of 0.02 they were pumped through the glass tubing enclosures at 340 ml min⁻¹ and light signals were measured with the integrated circuit microluminometer and recorded with time. Liquid samples were removed periodically to determine the absorbance and bioluminescence of the cultures. When cultures attained an O.D.₅₄₆ of 0.08, which corresponds to *ca* 9 × 10⁷ cells ml⁻¹, the cultures were challenged with various concentrations of sodium salicylate. For example, Fig. 3 shows the response of duplicate cultures after the exposure to 0.5 mg l⁻¹ of salicylate. After 25 min, the bioreporters from the two induced cultures began to produce a increased bioluminescence that was reproducibly detected by two different integrated circuits and a commercially available portable luminometer. The bioluminescence continued to increase until the culture became nutrient limited and energy starved (data not shown). The delay in the response time is principally attributed to mass transfer of salicylate into the cytoplasm of the cells and to a lesser extent, the expression of the genes responsible for bioluminescence. This increase in the rate of bioluminescence was dependent on the concentration of salicylate between 0.05 and 1 mg l⁻¹ and demonstrated saturation near 1 mg l⁻¹ (Fig. 4). The response times decreased from 45 to 20 min as the concentration was increased from 0.05 to 1 mg l⁻¹. We are currently using these and other benchmark data obtained with the glass tube enclosures to understand and enhance the analytical capabilities of the BBIC technology and for the development of different types of application-specific enclosures.

5.2 Off-chip wireless BBIC

Although CMOS technology can be used to create inexpensive electronic devices, the development of a few prototype integrated circuits can be quite costly. To dramatically reduce the cost of RF wireless communication testing for environmental analysis, a spread-spectrum BBIC

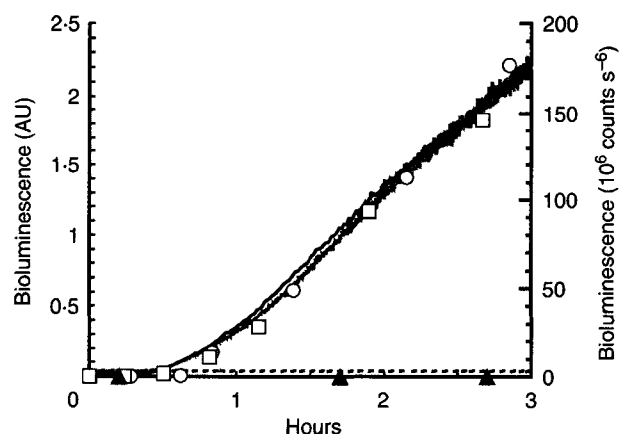


Fig. 3 Comparison of bioluminescence obtained from a bioreporter *Pseudomonas fluorescens* 5RL as measured on-line by integrated circuits (data shown as curves using the left axis) and off-line with a standard luminometer (data shown as symbols using the right axis). In the presented experiment, three cultures were grown with minimal salts medium in aerated flasks. When the cultures reached an O.D.₅₄₆ of 0.02, each culture was then pumped through a glass tube enclosure mounted onto a ceramic carrier that supported the integrated circuit. When two of the cultures reached an O.D.₅₄₆ of 0.08 (time equal to zero), enough salicylate was added to the two experimental flasks to produce a final concentration of 0.5 mg l⁻¹. Integrated circuit data from experimental flasks A and B are presented as the solid black and solid gray curves, respectively, whereas the standard luminometer data are presented using open squares and open circles, respectively. After ca 30 min, the bioluminescence from the experimental cultures increased rapidly, while the control culture, which was not exposed to salicylate, remained near background levels (flask C – black dash curve and black triangles). The BBIC responses are reported in arbitrary units (AU) and the luminometer responses are reported as counts per second

prototype was developed using commercially available off-chip components (Fig. 5). The prototype consisted of a BBIC chip mounted in an end-on orientation onto a printed circuit board that features biasing resistors and variable potentiometers to adjust BBIC operating parameters, a transmitter unit and a receiver unit. The chip and printed circuit board fit into an aluminum probe that was 9 cm in length and had a 2.5-cm diameter. The chip was sealed into the aluminum probe with PDMS. The end of the probe was sealed with an end-cap using an O-ring. The transmitter unit performs pulse interval timing measurements, in addition to data packaging and RS232 telemetry to a receiver that connects to the serial port of a computer-based data acquisition system. Timing and data packaging within the transmitter is performed by an ATMEL flash-programmable microcontroller (San Jose, CA, USA). The resultant wireless BBIC could reliably transmit data at distances over 500 ft for line of sight and 300 ft into a metal structured building. We are currently using this version of

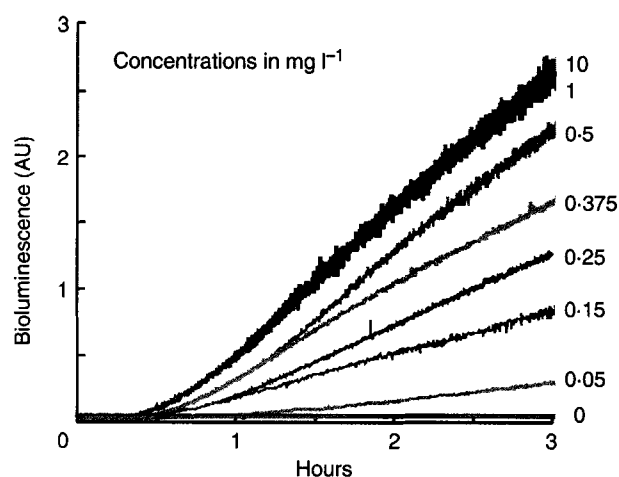


Fig. 4 Response curves for the bioreporter *Pseudomonas fluorescens* 5RL exposed to various concentrations of salicylate. Each bioluminescence curve was obtained using an aerated culture, a flow-through enclosure, and an integrated circuit detector operating under the conditions described in (Fig. 3). These results show that the detection limit is ca 0.05 mg l⁻¹ for a 45 min exposure and the bioluminescent response saturates at a concentration of ca 1 mg l⁻¹. The response time decreased to a minimum of 20 min at 1 mg l⁻¹ as the concentration was increased. The delay in the response time is probably caused by the slow mass transfer of salicylate into the cytoplasm of the bioreporters. Bioluminescence is reported in arbitrary units (AU)

the BBIC for remote monitoring until tested wireless chips become available.

Using the wireless BBIC probe, a liquid culture of *Ps. fluorescens* 5RL contained within the cap was exposed to 0, 0.05, 0.1, 0.5 and 5 mg l⁻¹ salicylate and the bioluminescence monitored with time (Fig. 6). The response of the 0.05 mg l⁻¹ was similar to background levels obtained with a culture that was not exposed to salicylate and the response of

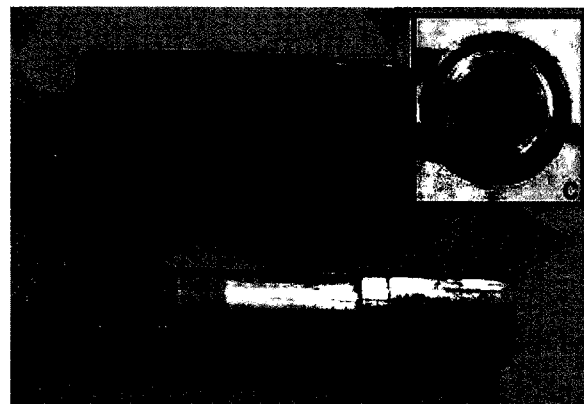


Fig. 5 Off-chip wireless BBIC probe (A) and transmitter box (B). The cap on the right end of the probe is removable and exposes the 2.2 × 2.2 mm integrated circuit (C)

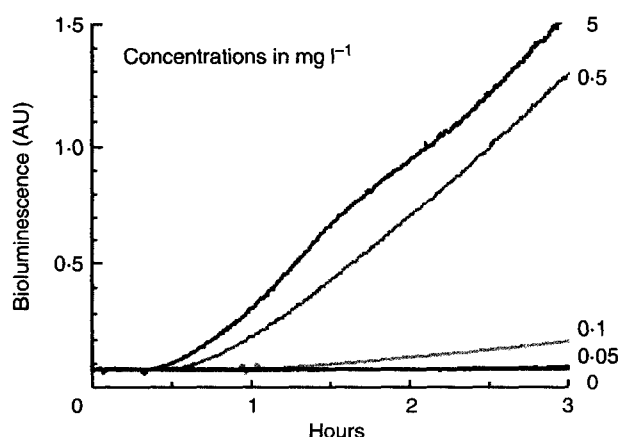


Fig. 6 Response curves in quiescent solutions for the bioreporter *Pseudomonas fluorescens* 5RL after exposure to various concentrations of salicylate. Briefly, cultures were grown to an O.D.₅₄₆ of 0.08 and exposed to 0, 0.05, 0.1, 0.5, and 5 mg l⁻¹ of salicylate and the bioluminescence continuously monitored with time using a wireless probe-style BBIC. Each culture was induced with salicylate at 0 h and immediately 1 ml was added to the enclosure. As the concentration of salicylate increased, the response times decreased from 55 min with a 0.1-mg l⁻¹ dose to 22 min with a 5 mg l⁻¹ exposure. The response times for each concentration were delayed over corresponding benchmark data that was obtained with cultures that were rotated for aeration (see Fig. 4). Data were collected at 20.0 ± 0.5°C. Bioluminescence is reported in arbitrary units (AU)

the 0.1 mg l⁻¹ exposure did not give a positive response until 55 min. An exposure to 5 mg l⁻¹ concentration demonstrated a more rapid response of 22 min. Comparison of these results with the benchmark data obtained with the aerated cultures indicates that the mass transfer of nutrients will play an important role in the design of the enclosures.

6. FUTURE CONSIDERATIONS

Successful remote environmental monitoring with a BBIC will require (i) a means of preserving bioreporters prior to use, (ii) a method to effectively resuscitate cells from storage and (iii) creation of a micro-environment that will maintain the bioreporters in a physiological state that allows a rapid response for possibly weeks to months under the conditions dictated by the environment. Living micro-organisms have been most successfully prepared for long-term storage by (i) liquid-drying and vacuum-sealing procedures, (ii) freeze-drying methods (Miyamoto-Shinohara *et al.* 2000) and/or (iii) direct freezing (Malik 1995; Malik 1998). For storage below 0°C, a cryoprotectant is typically added to preserve the cells during freezing and thereby enhance the recovery of viable cells. Liquid-drying and freeze-drying processes are used to prepare the bioreporter *V. fischeri* for storage with commercially available toxicity bioassay kits (Jennings

et al. 2001). Survival under desiccated conditions can require the use of specific storage compounds to avoid complete loss of water (Fedorov *et al.* 2000). Resuscitation of dried cells requires only hydration in an appropriate medium followed by an incubation time to remove any preservatives and to establish the proper physiological state for sensing the analyte. Storage and resuscitation may be facilitated by use of micro-environments that contain encapsulation matrices.

The micro-environment, a confined space that is defined by the enclosure and located near or on the integrated circuits (Fig. 1), can be filled with liquid cultures of bioreporters or encapsulation matrices that confine bioreporters within the pore spaces. Encapsulation matrices provide protection for the bioreporters and can contain nutrients and hydration to help sustain a particular bioreporter in a physiological state necessary for sensing. Several different types of encapsulation matrices have been used for the entrapment of nutrients and viable micro-organisms in thin films or beads (Simpson *et al.* 1998; D'Souza 2001). Many of these materials presently have drawbacks such as (i) preparation procedures that are toxic to the cells, (ii) analyte adsorption onto or absorption into the matrix, (iii) lack of mechanical strength in thin films of the materials, and (iv) unstable polymers that degrade with time. For example, sol gels (silica gels) are chemically inert and optically transparent materials that can be mixed with cells and dried to form an extremely porous and rigid glass-like cage encapsulating the micro-organisms (e.g. Coiffier *et al.* 2001; Premkumar *et al.* 2002). However, when sol gels are formed with alkoxide precursors, methanol is formed which may be toxic to the cells. Coiffier *et al.* (2001) have suggested that an aqueous precursor, which forms sodium ions instead of methanol, may be used to minimize the toxic effect.

Flickinger and colleagues have devised a method to entrap viable micro-organisms in partially coalesced latex coatings that are nontoxic to many micro-organisms (Lyngberg *et al.* 1999a; Lyngberg *et al.* 2001) and have used the latex encapsulation with a *mer-luxCDABE E. coli* strain (Lyngberg *et al.* 1999b). These coatings have many advantages over other types of encapsulation matrices because they (i) can be made very thin (20–100 µm thick) to increase response times, (ii) are mechanically flexible but strong, (iii) can support large amounts of biomass (2 × 10¹¹ cells ml⁻¹), (iv) yield high cell viability after long-term storage at 4 and -20°C and (v) can even be stored (14 days) after drying and rehydration.

We are currently using the BBIC test systems to examine different combinations of encapsulation matrices and novel enclosure configurations and to create micro-environments for potential long-term monitoring. This research is developing BBIC systems that protect and contain the bioreporters and provide nutrients to maintain the cells in a

physiological state that is necessary for detecting an analyte of interest. This testing is designed to develop a bioreporter and micro-environment that provides bioluminescent signals with the optimal stability, reproducibility, repeatability, response times, detection limits, sensitivity, dynamic ranges, precision and accuracy for a given application. The results of this research will advance our fundamental understanding of interfacing bioluminescent bioreporters with integrated circuits to allow the development of early-warning, semi-quantitative and potentially quantitative biosensors for monitoring of real-world environments.

7. CONCLUSIONS

Bioreporter technology is evolving behind the wave of genomic and proteomic information that is being generated at an astounding pace. Whole genomic sequences of a number of micro-organisms are currently available (Nelson *et al.* 2000) and the complex interrelationships of global gene regulatory systems are being unraveled. Promoter-reporter gene fusions harness the ability of micro-organisms to sense their environments by intercepting intracellular messages that are then transformed into detectable signals that can be used to determine the presence and/or the concentration of analytes or physical changes in an environment. These intracellular messages, in the form of gene regulatory systems, can control the expression of an individual gene or genes that are part of a larger global response. The choice of the gene regulatory system that controls the expression of the reporter genes ultimately affects many of the analytical characteristics of a BBIC sensor, including selectivity, response times and detection limits.

Bacterial bioluminescence using the entire reporter gene cassette is widely utilized, as the substrates are, or can be formed from, simple metabolites. Although most widely used to construct Gram-negative bioreporters, the Lux A, B, C, D, and E genes have been expressed in the Gram-positive *Staphylococcus aureus* (as the lux ABCDE cassette) (Francis *et al.* 2000) and in the yeast *Saccharomyces cerevisiae* (Gupta *et al.* 2003), allowing further expansion of the lux reporter gene technology.

The attractiveness of bioluminescent bioreporter signaling for remote whole-cell bioreporters is related to the fact that the bacteria use biochemical energy to generate the signal with no additional power drain on the battery. Power consumption often is a limiting factor for the lifetime of small inexpensive devices that are remotely deployed. Furthermore, bioluminescence can be detected without the complications associated with optical sources or filters. To further reduce power consumption, sensitive photodiode-based integrated circuits realized in CMOS that can detect 490 nm light have been produced and tested. Under laboratory conditions, we have demonstrated that the

photodiodes can detect bioluminescence from bioreporters in a concentration-dependent manner.

A version of the chip has been designed to incorporate an on-chip temperature sensor to facilitate the development of real-time temperature compensation. Future versions of the chip will utilize an array of photodiodes for the detection of multiple signals from multiple bioreporters on a single chip. Multi-photodiode chips will be important for the development of biosensors that incorporate control strains, different promoter-luxCDABE fusions for multi-analyte detection and possibly a genome-wide BBIC application.

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A Chromosomally Based *tod-luxCDABE* Whole-Cell Reporter for Benzene, Toluene, Ethylbenzene, and Xylene (BTEX) Sensing

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A *tod-luxCDABE* fusion was constructed and introduced into the chromosome of *Pseudomonas putida* F1, yielding the strain TVA8. This strain was used to examine the induction of the *tod* operon when exposed to benzene, toluene, ethylbenzene, and xylene (BTEX) compounds and aqueous solutions of JP-4 jet fuel constituents. Since this system contained the complete *lux* cassette (*luxCDABE*), bacterial bioluminescence in response to putative chemical inducers of the *tod* operon was measured on-line in whole cells without added aldehyde substrate. There was an increasing response to toluene concentrations from 30 µg/liter to 50 mg/liter, which began to saturate at higher concentrations. The detection limit was 30 µg/liter. There was a significant light response to benzene, *m*- and *p*-xylenes, phenol, and water-soluble JP-4 jet fuel components, but there was no bioluminescence response upon exposure to *o*-xylene. The transposon insertion was stable and had no negative effect on cell growth.

Due to the widespread use of petroleum products and the current regulations requiring underground storage tanks to be upgraded, replaced, or closed by December 1998 (4), the number of petroleum-contaminated sites has abounded. Of particular concern for drinking water quality are the more water-soluble components, benzene, toluene, ethylbenzene, and xylenes (BTEX). Natural attenuation, which relies on in situ biodegradation of pollutants, has received a large amount of attention, especially for petroleum contaminants (15). While microorganisms capable of biodegradation of BTEX compounds are usually present at these sites, there is a need to know whether or not conditions are favorable for biodegradation to occur. A recent approach to determine whether compounds are bioavailable and what conditions are favorable for degradation is the use of whole-cell bioluminescent reporters (9).

Bioluminescent reporters have been widely used for the real time nondestructive monitoring of gene expression. Heitzer et al. (8) developed a quantitative assay for naphthalene bioavailability and biodegradation by using a *nah-lux* reporter strain constructed by King et al. (13) and expanded its use as an on-line optical biosensor for application in groundwater monitoring (10). Other *lux* fusions have been constructed for monitoring the expression of catabolic genes, including those for degradation of isopropylbenzene (21) and toluene (1, 5). *lux* fusions have also been constructed for monitoring heat shock gene expression (24, 25), oxidative stress, (3), the presence of Hg(II) (20), and alginate production (26). In all of these cases, the *lux* fusions were plasmid based and were constructed by placing the promoter of interest in front of the promoterless *lux* genes from *Vibrio fischeri* contained in pUCD615 (18).

In this study, a strategy was pursued to introduce a single copy of the *lux* fusion into the bacterial chromosome via a transposon delivery system. A mini-Tn5 delivery vector con-

structed by Herrero et al. (11) provided the basic model for this work. By this approach, a *tod-lux* fusion was constructed and introduced into *Pseudomonas putida* F1 to examine the induction of the *tod* operon when exposed to BTEX compounds and aqueous solutions of JP-4 jet fuel constituents. Since this system contains the complete *lux* cassette (*luxCDABE*), bacterial bioluminescence was measured on-line in whole cells without addition of an aldehyde substrate. The resultant strain was also evaluated for its stability and fitness compared to those of the wild-type strain, F1.

Organisms and culture conditions. The strains used in these experiments are shown in Table 1. All cultures were grown at 28°C with appropriate antibiotic selection, except for *Escherichia coli* strains, which were grown at 37°C.

DNA isolation and manipulation. Large-scale plasmid DNA isolation was done by a modified alkaline lysis protocol (16). Chromosomal DNA was prepared by the protocol outlined by Ausubel et al. (2). All DNA preparations were further purified by CsCl-ethidium bromide ultracentrifugation (19). DNA modifications and restriction endonuclease digestions were performed as outlined by Sambrook et al. (19).

Transposon and plasmid construction. The transposon mini-Tn5Km^r was constructed with two 58-base oligonucleotides 5' and 3' with respect to the kanamycin resistance gene (Km^r) in pCR II (Invitrogen, San Diego, Calif.) (I end, 5'GGGCGCTAGCGAAATGTTGACTGTCTCTTGATCAGATCTTTCAATTTCAGAAGAACTCG3'; O end, 5'CGAATTCTGACTCTTATACACAAGTTCTAGATTGCGGCCGCTTGGTTAAAAATGAGC3'). Oligonucleotides were synthesized with a Beckman Oligo 1000 DNA synthesizer (Palo Alto, Calif.). Base substitutions were made to generate both I and O insertion sequences as well as unique *NotI* and *XbaI* sites inside the transposon for cloning. An extra adenine was mistakenly added between the *NotI* and *XbaI* sites in the O primer, but it did not affect the construction. Primers were used to amplify the kanamycin resistance gene from pCR II by using touchdown PCR (7). The manufacturer's protocol was used with the following thermocycler conditions. Initial denaturation at 94°C for 5 min, followed by five cycles of denatur-

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TABLE 1. Strains and plasmids used in this study

Plasmid or strain	Relevant genotype or characteristics	Source or reference
Plasmids		
pDTG514	pGem3Z with a 2.75-kb <i>EcoRI-SmaI</i> fragment from pDTG350 containing the <i>tod</i> promoter, P_{tod} ; Ap ^r	14
pUCD615	Promoterless <i>luxCDABE</i> cassette containing <i>ori</i> pSa and <i>ori</i> pBR322; Ap ^r Km ^r	18
pKK223-3	Expression vector containing the 5S ribosomal terminator <i>rrnB</i> T ₁ T ₂	Pharmacia
pBSKS	pBluescript II KS ⁺ with MCS <i>KpnI-SacI</i> ; Ap ^r	Stratagene
pBSMCS(-)	pBluescript without MCS (<i>Bss</i> HII- <i>Bss</i> HII fragment removed); Ap ^r	This study
pLJS	pBSMCS(-) with added <i>XbaI</i> , <i>NheI</i> , <i>AvrII</i> , and <i>SpeI</i> sites; Ap ^r	This study
pLJS- <i>tod</i>	pLJS containing the 1.8-kb <i>SmaI-XhoI</i> <i>tod</i> promoter fragment from pDTG514; Ap ^r	This study
pLJS- <i>lux</i>	pLJS containing the 8.35-kb <i>KpnI-PstI</i> <i>luxCDABE</i> cassette from pUCD615; Ap ^r	This study
pLJST2	pLJS containing the 0.77-kb <i>HindIII-HincII</i> fragment from pKK223-3 cloned into <i>HindIII-SmaI</i> site; Ap ^r	This study
pUC18Not	Cloning vector containing MCS flanked by <i>NotI</i> sites; Ap ^r	11
pUC18Not- <i>lux</i>	Contains the 8.35-kb <i>XbaI-PstI</i> fragment from pLJS- <i>lux</i> ; Ap ^r	This study
pUC18Not- <i>todlux</i>	Contains the 1.8-kb <i>SpeI-XhoI</i> fragment from pLJS- <i>tod</i> ; Ap ^r	This study
pUT	5.2-kb cloning vector containing <i>mob</i> RP4, <i>ori</i> R6K and Tn5 <i>tnp</i> lacking <i>NotI</i> sites; Ap ^r	11
pCR II	3.9-kb cloning vector for PCR products with 3' A overhangs; Ap ^r Km ^r	Invitrogen
pUTK209	pCR II containing mini-Tn5KmNX with unique <i>NotI</i> and <i>XbaI</i> sites; Ap ^r Km ^r	This study
pUTK210	pUT containing mini-Tn5KmNX; Ap ^r Km ^r	This study
pUTK211	pUT/mini-Tn5KmT2 containing the 0.8-kb <i>NotI-AvrII</i> <i>rrnB</i> T ₁ T ₂ fragment; Ap ^r Km ^r	This study
pUTK214	pUT/mini-Tn5Km <i>tod-lux</i> containing the 10.2-kb <i>NotI-XbaI</i> fragment from pUC18Not- <i>todlux</i> ; Ap ^r Km ^r	This study
Strains		
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA</i> <i>supE44</i> λ ⁻ <i>thi-1</i> <i>gyrA96</i> <i>relA1</i>	Gibco BRL
S17-1(λ pir)	λ pir <i>recA</i> <i>thi</i> <i>pro</i> <i>hsdR</i> M ⁺ RP4:2-Tc:Mu:Km Tn7Tp ^r Sm ^r ; mobilizing strain for pUT/mini-Tn5 derivatives	6
INV α F ^r	Strain used with TA cloning vector, pCR II; F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA</i> <i>supE44</i> λ ⁻ <i>thi-1</i> <i>gyrA96</i> <i>relA1</i>	Invitrogen
<i>P. putida</i>		
F1	Contains a chromosomally encoded <i>tod</i> operon for toluene degradation	28
TVA8	F1 containing a mini-Tn5Km <i>tod-lux</i> insertion in the chromosome; Km ^r	This study

ation at 94°C for 1 min, 72°C annealing for 1 min, and 72°C extension for 2 min. The annealing temperature was then lowered 5°C every five cycles until 42°C, at which point, eight cycles were run, followed by a final extension of 15 min at 72°C. The resultant PCR fragment was cloned into the transposon delivery vector pUT, generating pUTK210. The cloning vector pLJS was constructed from pBluescript II (KS) (Stratagene, LaJolla, Calif.) by cleavage with *Bss*HII removing the multicloning site (MCS). The resultant plasmid was named "pBSMCS(-)." Two oligonucleotides (a 47-mer and a 44-mer) (*KpnI* end, 5'CCAAGCGCGCAACTAGTCTAGACTAAAGCTAGCCTAGGCTGGGATCC3'; *SacI* end, 5'GTGAGCGCGCGTAATACGAGCTAGCCTAGGGCGAATTGGAGCA C3') were synthesized to regenerate the MCS and add the restriction sites *XbaI*, *NheI*, *SpeI*, and *AvrII*. The orientation of the added sites can be seen in Fig. 1. The new MCS was amplified from pBluescript II (KS) by using the manufacturer's protocol with the following thermocycler conditions. Initial denaturation was at 94°C for 5 min, followed by 38 cycles of denaturation at 94°C for 30 s, annealing at 42°C for 1 min, extension at 72°C for 30 s, and final extension at 72°C for 15 min. The amplified fragment was cleaved with *Bss*HII, ligated into pBSMCS(-), and transformed into DH5 α . A portion of pLJS was sequenced, confirming the base substitutions and integrity of the MCS, with an Applied Biosystems model 373A sequencer (Foster City, Calif.). Plasmid pLJST2 was generated

by directional cloning of the 0.8-kb *HindIII-HincII* fragment containing the 5S ribosomal *rrnB* T₁T₂ transcription terminator from pKK223-3 (Pharmacia, Piscataway, N.J.) into pLJS cleaved with *HindIII* and *SmaI*. The *NotI-AvrII* terminator fragment from pLJST2 was subsequently cloned into the *NotI-XbaI* site of pUTK210, yielding pUTK211 containing mini-Tn5KmT2. This allowed for the subsequent destruction of the *XbaI* site by heterologous ligation and the regeneration of the *NotI* and *XbaI* unique sites downstream of the terminator. Mini-Tn5Km*tod-lux* (pUTK214) was generated by directional cloning of the 10.2-kb *NotI-XbaI* *tod-lux* fragment from pUC18Not-*todlux* (Table 1) into the *NotI-XbaI* site of pUTK211. Both the insert and vector DNA were purified by agarose gel electrophoresis and electroeluted prior to cloning. Electrocompetent *E. coli* S17-1(λ pir) cells were prepared and ligations were electroporated as outlined by the manufacturer (BTX, San Diego, Calif.). All other plasmids and relevant constructs are described in Table 1.

Strain construction. Plasmid pUTK214 was conjugated into *P. putida* F1 from *E. coli* S17-1(λ pir) as previously described (6). Strains carrying transposon insertions were selected on *Pseudomonas* isolation agar (Difco, Detroit, Mich.) supplemented with 50 μ g of kanamycin/ml. Colonies which produced light upon exposure to toluene were grown in mineral salts media (MSM) (23) with toluene vapor to ascertain that the transposon had not inserted into a gene required for cell

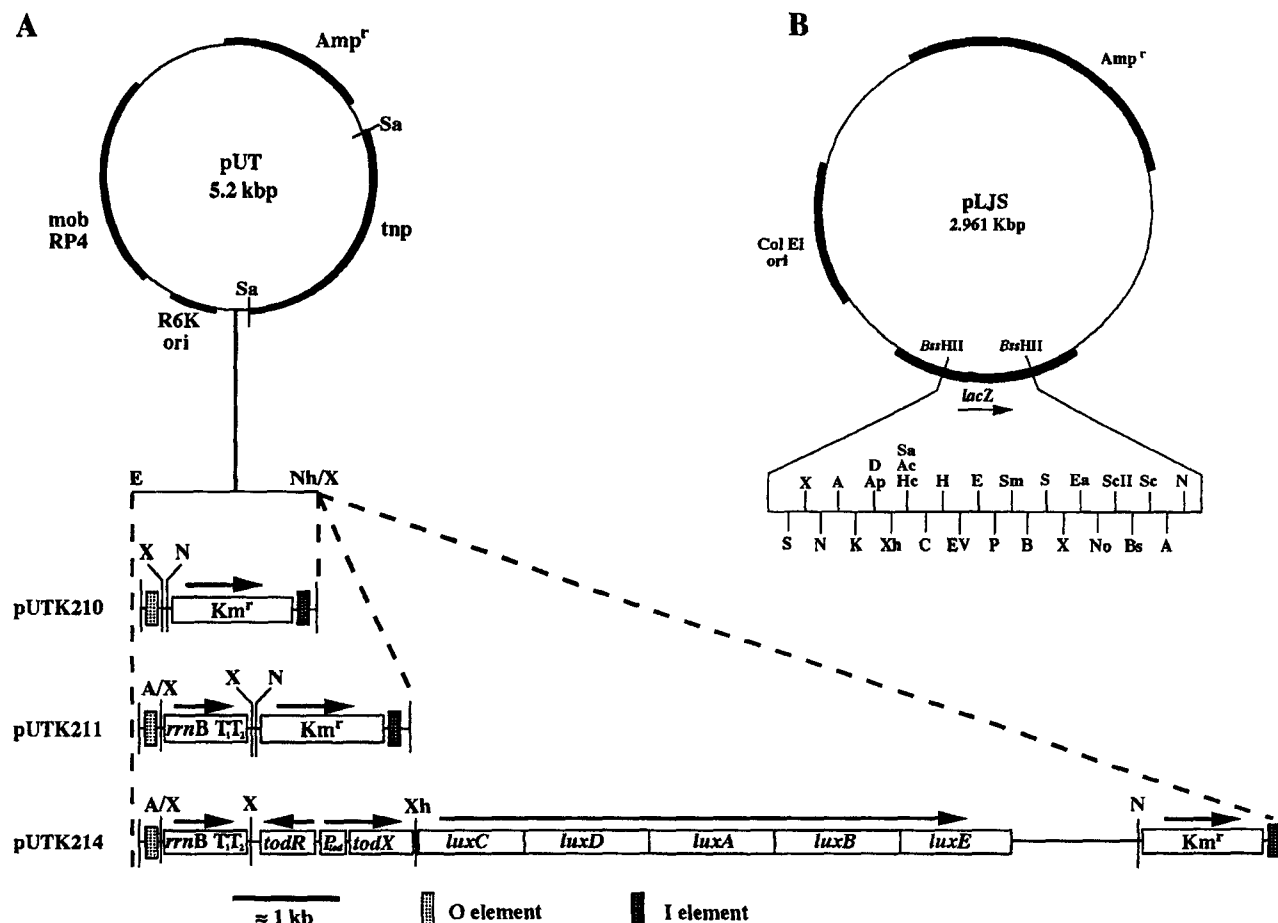


FIG. 1. (A) Construction of mini-Tn5Kmtd-*lux*. A/X and Nh/X represent the *AvrII-XbaI* and *NheI-XbaI* heterologous cloning sites, respectively. N, *NotI*; Sa, *Sall*; X, *XbaI*. (B) Cloning plasmid pLJS with unique restriction sites. A, *AvrII*; Ac, *AccI*; Ap, *ApaI*; B, *BamHI*; Bs, *BstXI*; C, *Clal*; D, *DraII*; E, *EcoRI*; Ea, *EagI*; EV, *EcoRV*; H, *HindIII*; Hc, *HincII*; K, *KpnI*; Nh, *NheI*; P, *PstI*; S, *SpeI*; Sc, *SacI*; ScII, *SacII*; Sm, *SmaI*; Xh, *XhoI*.

growth and also to evaluate their performance as bioreporters in liquid, growing-cell assays (8). A strain designated TVA8 was selected for further study and subjected to DNA-DNA hybridization to verify transposition, as opposed to recombination, by using a ^{32}P -labeled probe specific for the Tn5 transposase (*tnp*) contained on pUT. Equal target amounts of *luxA*, *todC*, and *tnp* DNA were loaded onto a Biotrans nylon membrane (ICN, Irvine, Calif.) by using a Bioslot blot apparatus (Bio-Rad, Hercules, Calif.) according to the manufacturer's protocol. The blot consisted of chromosomal DNA from F1, TVA8, and the aforementioned controls. The DNA was loaded in triplicate, the blot was subdivided, and each separate blot was hybridized with either *luxA*, *todC*, or *tnp* PCR-generated ^{32}P -labeled DNA probes. Blots, hybridized and washed as previously described (1), verified that TVA8 contained *luxA* and *todC* but not *tnp* (data not shown). The negative transposase result confirmed that transposition had occurred.

Stability assays. Batch stability assays were performed by transfer of 1 ml of a 100-ml overnight culture grown in Luria-Bertani (LB) broth with 50 μg of kanamycin/ml (LBK₅₀) to a 250-ml Erlenmeyer flask with toluene used as the sole carbon source (supplied as vapor). One milliliter of culture was transferred every day for 5 days to flasks containing 100 ml of MSM supplied with toluene vapor (without K₅₀). Assays were performed in triplicate. Before each transfer, cells were plated on selective (LBK₅₀) and nonselective (LB) media to ascertain

loss of kanamycin resistance resulting from deletion or excision of the transposon. Colonies were subjected to colony hybridization with a 295-bp *luxA* DNA probe (12). Stability was also assayed in continuous culture with a New Brunswick Bio Flow fermentor (Edison, N.J.) with a 370-ml vessel operated at 28°C at 180 rpm. The feed consisted of MSM supplemented with toluene at approximately 100 mg/liter at a flow rate of 1.0 ml/min. Toluene was fed by simultaneously adding toluene-saturated MSM at a flow rate of 0.2 ml/min and MSM at a flow rate of 0.8 ml/min by using FMI metering pumps (Oyster Bay, N.Y.). The fermentor was operated for 14 days (100 generations) with daily bioluminescence and optical density (OD) measurements. Plate counts (at 7 and 14 days) from selective (LBK₅₀) and nonselective (LB) media were compared to determine if the kanamycin marker was being lost, and *luxA* colony blot hybridization was performed to confirm that all colonies contained the *lux* transposon insert. In batch and chemostat stability studies, TVA8 did not demonstrate instability when subjected to the same evaluation. From batch assays, the selective/nonselective plate count ratio was 1.12 ± 0.13 after five daily transfers, and all colonies hybridized with the *luxA* probe. After a 14-day continuous cultivation, the selective/nonselective plate count ratio was 1.05 ± 0.13 , and all colonies from selective and nonselective plates were *lux* positive.

Comparison of growth of TVA8 and F1 on toluene. To examine the effect of bioluminescence on the fitness of TVA8, growth curves of TVA8 and F1 were obtained by growing cells

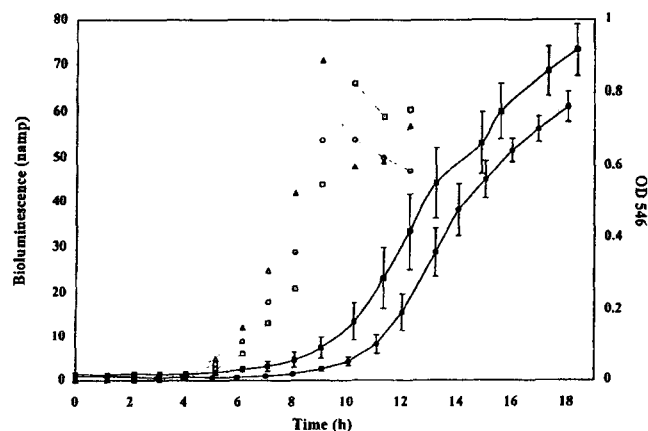


FIG. 2. Bioluminescence and growth of TVA8 and growth of F1 on toluene vapor under batch conditions. ○, □, and △ represent individual replicates of bioluminescence readings over time. The solid squares (TVA8) and circles (F1) represent the average OD₅₄₆ of three replicates.

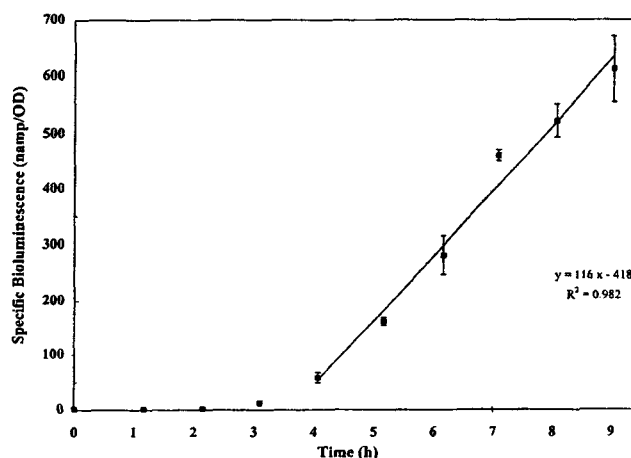


FIG. 3. Specific bioluminescence of TVA8 grown on toluene vapor. The regression line equation is $y = 116x - 418$ ($r^2 = 0.982$). (The first four time points were not included in the linear regression because the organisms were in lag phase.)

in 100 ml of MSM in 250-ml Erlenmeyer flasks with toluene vapor supplied as the sole carbon source. Flasks were inoculated from a fresh overnight culture, grown to an OD at 546 nm (OD₅₄₆) of 1.0 in 100 ml of LB, washed twice in 100 ml of MSM, and resuspended in 100 ml of MSM. A 1-ml aliquot of this suspension was added to the toluene flasks. The cultures were shaken at 200 rpm at 28°C and sampled approximately every hour. The OD₅₄₆ was measured for each culture, and rates of increase in OD were determined from the linear portion of the curves. Growth curves for TVA8 and the parent strain F1 on toluene vapor are shown in Fig. 2. The curves show similar shapes with different lag times for TVA8 and F1 that can be attributed to slightly different inoculum concentrations. Rates were computed from the slopes of the linear portion of the growth curve for both strains. The average rates of increase in OD for F1 and TVA8 were (2.1 ± 0.3) and $(2.2 \pm 0.3) \text{ min}^{-1} \times 10^{-3}$, respectively and were not statistically different ($\alpha = 0.05$). These results demonstrate that the bioluminescence reactions do not appear to affect cell growth.

Bioluminescence of TVA8 was measured during growth on toluene and is shown along with the cell density data in Fig. 2. The graph shows that there is a relationship between an increase in biomass and an increase in light production. At higher cell densities, cells likely became limited for oxygen, resulting in decreased bioluminescence. Specific bioluminescence (nanoamperes/OD₅₄₆) of TVA8 versus time shows an increase in specific bioluminescence (Fig. 3). This suggests that a steady state of luciferase in the cell is not obtained in this time frame and that luciferase is accumulating (Fig. 3).

Bioluminescence response of TVA8 to toluene, BTEX compounds, and JP-4 jet fuel. Bioluminescence assays were conducted as described by Heitzer et al. (8). Aqueous solutions of toluene, benzene, ethylbenzene, phenol, isomers of xylene, and JP-4 jet fuel constituents were prepared by adding pure hydrocarbon or JP-4 to MSM in a 1:10 (vol/vol) ratio. The solutions were placed on a rotary shaker for 24 h. After phase separation, aqueous-phase aliquots were added to test vials. Final concentrations of dissolved toluene in growing-cell assays ranged from 0 to 50 mg/liter (based on water solubility). The final concentration of the other hydrocarbons was 50 mg/liter (based on their water solubility), and the percentage of JP-4-saturated MSM in the test samples was 2%. Vials containing test solutions and cells were shaken at 150 rpm on a rotary

shaker, and bioluminescence was measured every 30 min. Sample vials were placed in a light-tight box, and light output was measured with a liquid light pipe and an Oriel photomultiplier and digital display (models 77340 and 7070; Stratford, Conn.) (8). The light detection methods for continuous culture and growth curves were similar, except that the light-tight box was modified to hold a cuvette, allowing for light measurements and OD readings to be taken consecutively.

In preliminary experiments, an incubation time of 2 h was shown to provide a consistent light response and signal intensity. After 2 h, the final OD₅₄₆ was measured, and values were expressed as specific bioluminescence (nanoamperes/OD₅₄₆). An increase in bioluminescence was observed to correlate with increasing toluene concentrations (Fig. 4). The bioluminescence response to toluene concentrations over the range of 5 to 20 mg/liter was linear, with specific bioluminescence values of 133 to 228 nA/OD₅₄₆. The fold increase in light response for concentrations above 20 mg/liter was less, showing a specific bioluminescence value of 290 nA/OD₅₄₆ at 50 mg of toluene per liter. The overall bioluminescence response curve exhib-

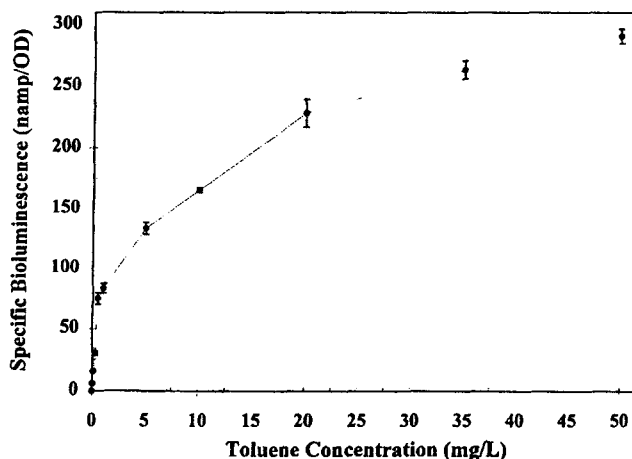


FIG. 4. Bioluminescence response of TVA8 to increasing concentrations of toluene after a 2-h exposure. Values are averages of three replicates and have been normalized to the cell density (OD₅₄₆).

TABLE 2. Effect of BTEX, phenol, and JP-4 constituents on the bioluminescence response of TVA8

Treatment ^a	Exposure time (h)	Specific bioluminescence (nA/OD) ^b
Buffer (control)	2	0.2 ± 0.1
Toluene	2	234 ± 7
Benzene	2	242 ± 9
Ethylbenzene	2	1.0 ± 0.2
	4	47 ± 6 ^c
<i>o</i> -Xylene	2	0.5 ± 0.1
<i>m</i> -Xylene	2	38 ± 3
<i>p</i> -Xylene	2	24 ± 2
Phenol	2	70 ± 2
JP-4	2	93 ± 4

^a Final concentration for BTEX and phenol treatments was 50 mg/liter (based on water solubilities), and they were added as a hydrocarbon-saturated MSM solution. The final percentage of water-soluble JP-4 constituents was approximately 2%.

^b Values are averages ± standard deviations of three replicate samples. Values were normalized to the final cell density (OD₅₄₆).

^c The value for the 4-h reading was measured from a similar but separate experiment.

ited Michaelis-Menten kinetics, showing saturation at higher toluene concentrations. The toluene detection limit was determined to be 30 µg/liter (threefold increase over background bioluminescence). There was a significant light response to benzene, *m*- and *p*-xylenes, phenol, and JP-4 (Table 2). The same concentrations of toluene and benzene (50 mg/liter) resulted in a similar light response. There was no increase in bioluminescence upon exposure to *o*-xylene. The light response due to JP-4 was significantly greater than the additive responses for JP-4 components (i.e., BTEX compounds) present at their estimated concentrations in water saturated with JP-4 (22). The increased response may be the result of induction due to other components of JP-4 which were not tested. A significant light response was observed for ethylbenzene after 4 h. After a 2-h incubation, the cell densities for the ethylbenzene treatments were significantly less than those for the other samples, indicating that there may have been a toxicity effect. Further experiments showed that 50 mg of ethylbenzene per liter would induce the bioluminescence response without a lag period when cells were previously grown on ethylbenzene and then subjected to growing-cell assays.

Conclusions. The majority of bioluminescent reporter systems currently being used are the result of cloning of a promoter in front of either a promoterless *luxAB* or *luxCDABE* gene cassette and transfer of the plasmid construct into the strain that contained the particular promoter. Plasmid-based systems have obvious drawbacks, such as the need for constant selective pressure to ensure plasmid maintenance (17). Another important consideration is plasmid copy number. In a positively regulated system, copy number can negatively effect gene expression. Multiple copies of the promoter binding region for the regulatory protein on the plasmid compete with the binding site on the chromosome, causing less expression of the operon being studied (27). This negative effect is important

when *lux* fusions are used for on-line monitoring of bacterial processes.

TVA8 was capable of growth on MSM with toluene as a sole carbon source, demonstrating that the transposon insertion did not disrupt a gene necessary for growth. Furthermore, TVA8 did not show loss of the transposon insertion or loss of bioluminescence after 100 generations in continuous culture or five successive transfers in batch culture without antibiotic selection. These results indicate that selective pressure is not necessary for strain integrity. This stability is important, since it eliminates the need for antibiotic selection, which, if required, would exclude the use of this bioreporter in situ. TVA8 was also compared to the wild-type strain, F1, to ascertain whether or not the bioluminescent reporter incurred a significant metabolic demand on the cell, as well as whether the site of transposition was a hindrance to the cell. No difference in growth between the two strains was seen, suggesting that neither the insertion site nor the *lux* fusion was a significant handicap to the cell.

The *tod-lux* reporter was highly sensitive, detecting 30 µg of toluene/liter. This bioreporter also showed a very low background level of bioluminescence (less than 1 nA/OD₅₄₆), demonstrating its usefulness for detecting toluene present at low concentrations in aqueous solutions. Significant light levels were observed for very low ODs (Fig. 2).

TVA8 can be described as a generalized BTEX bioreporter rather than simply a toluene bioreporter, since it was responsive to benzene, ethylbenzene, and *m*- and *p*-xylene and can therefore be used as a bioreporter for hydrocarbon contamination for fuels containing BTEX compounds. TVA8 can also be used for on-line monitoring of trichloroethylene cometabolism, since the *lux* and *tod* operons are under the same regulation (toluene dioxygenase catabolizes trichloroethylene).

Bioluminescent reporters may have great potential for field use applications, since they can provide on-line and nondestructive analyses of gene expression as well as detection of chemical contaminants. The development of stable transposon insertions of *lux* reporter gene fusions into environmental isolates expands the utility of bioreporter strains for in situ sensing of gene expression.

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